DCES NOT CIRCULATE

Comp

UNIVERSITY OF MICHIGAN
JUL 23 1954
MEDICAL LIBRARY

The Journal of LABORATORY and CLINICAL MEDICINE

VOL. 44 JULY, 1954 NO. 1

The Official Publication of the Central Society for Clinical Research

Clinical and Experimental

Observations in Air Force Recruits of Streptococcal Diseases and Their Control With Orally Administered Penicillin. Stanley H. Bernstein, Captain, USAF (MC), Harry A. Feldman, M.D., O. F. Harper, Jr., Captain, USAF (MSC), W. H. Klingensmith, First Lieutenant, USAF (MSC), and Jerome A. Cantor, Captain, USAF (MC), Syracuse, N. Y. ---- 1

Bacteriophage Typing of Staphylococci. I. Technique and Patterns of Lysis. George Gee Jackson, M.D., Harry F. Dowling, M.D., and Mark H. Lepper, M.D., Chicago, Ill. ---- 14

Bacteriophage Typing of Staphylococci. II. Epidemiologic Studies Among Patients, Household Contacts, and Hospital Personnel. George Gee Jackson, M.D., Harry F. Dowling, M.D., and Mark H. Lepper, M.D., Chicago, Ill. ---- 29

Bacteriophage Typing of Staphylococci. III. Relationship to Antibiotic Sensitivity and Resistance. George Gee Jackson, M.D., Mark H. Lepper, M.D., and Harry F. Dowling, M.D., Chicago, Ill. ---- 41

A Simple Procedure for the Identification of the Genus Salmonella by Means of a Specific Bacteriophage. William B. Cherry, Ph.D., Betty R. Davis, B.A., Philip R. Edwards, Ph.D., and Ralph B. Hogan, M.D., Atlanta, Ga. ---- 51

Influence of Repeated Histoplasmin Skin Tests on Precipitins and Complement-Fixing Antibodies. S. B. Salvin, Ph.D., R. W. Weber, M.D., D. B. Lackman, Ph.D., Jane Nishio, M.S., and Gladys Menges, R.N., Hamilton, Montana, and Kansas City, Kan. ---- 56

Determination of Iron in Small Amounts of Serum and Whole Blood With the Use of Thiocyanate. Hugh W. Josephs, M.D., Baltimore, Md. ---- 63

Metabolic and Histologic Studies in Patients With and Without Liver Disease Receiving Chloramphenicol and Oxytetracycline. William W. Faloon, M.D., Syracuse, N. Y. With the technical assistance of Joan W. Noll, B.S., and Kathleen Collins, B.S. ---- 75

Fecal Fat on an Essentially Fat-Free Diet. George T. Lewis, Ph.D., and Helen C. Partin, M.S., Miami, Fla. ---- 91

Acquired Antithromboplastinogenemia (Hemophilia-Like Disease): With Special Reference to Its Diagnosis. Armand J. Quick, Ph.D., M.D., Einar R. Daniels, M.D., and Clara V. Hussey, M.S., Milwaukee, Wis. ---- 94

(Contents continued on inside front cover)

PUBLISHED BY THE C. V. MOSBY COMPANY, ST. LOUIS 3, MISSOURI

Copyright 1954 by The C. V. Mosby Company

Contents—*(Continued from front cover)*

The Hemodynamic and Metabolic Response of the Human Hypertensive Kidney to a Standard Dose of 1-Hydrazinophthalazine (Hydralazine). Archer P. Crosley, Jr., M.D., George G. Rowe, M.D., and Charles W. Crumpton, M.D., Madison, Wis. 104

Human Serum Albumin Tagged With I^{131} in Patients With Ascites Caused by Abdominal Carcinomatosis and Portal Cirrhosis: The Rates of Interchange Between the Vascular Compartment and Peritoneal Cavity. Malcolm P. Tyor, M.D., Oak Ridge, Tenn. ---------- 110

The Relationship of Vitamin B_{12} and Folic Acid in Megaloblastic Anemias. H. O. Nieweg, M.D., J. G. Faber, Ph.D., J. A. de Vries, and W. F. Stenfert Kroese, Groningen, Netherlands ---------- 118

Incidence of Hemoglobin C Trait in 505 Normal Negroes: A Family With Homozygous Hemoglobin C and Sickle-Cell Trait Union. Rose G. Schneider, Ph.D., Galveston, Tex. ---------- 133

Laboratory Methods

The Cheek Pouch of the Chinese Hamster (Cricetulus griseus) For Cinephotomicroscopy of Blood Circulation and Tumor Growth. George P. Fulton, Ph.D., Brenton R. Lutz, Ph.D., Donald I. Patt, Ph.D., and George Yerganian, Ph.D., Boston, Mass. With the technical assistance of Kenneth A. Arendt, A.M., Roma Kagan, A.B., and Frederick W. Maynard, A.B. ---------- 145

An Improved Method for Multiple Rapid Determinations of Arterial Blood pH. Duncan A. Holaday, M.D., New York, N. Y. ---------- 149

Simultaneous Determination of Sucrose and Inulin in Biologic Fluids. M. Kendall Young, Jr., M.A., and John F. Prudden, M.D., Med. Sc. D., Fort Sam Houston, Tex. ---------- 160

The Determination of Cyanide in Biologic Fluids by Microdiffusion Analysis. Milton Feldstein, M.A., and Niels C. Klendshoj, M.D., Buffalo, N. Y. ---------- 166

Editor

CLAYTON G. LOOSLI, M.D.

Associate Editor

ROBERT H. EBERT, M.D.

University of Chicago School of Medicine
950 E. 59th Street
Chicago 37, Illinois

Board of Editors

S. HOWARD ARMSTRONG, JR., M.D.
University of Illinois, Chicago

FRANK H. BETHELL, M.D.
University of Michigan, Ann Arbor

RICHARD B. CAPPS, M.D.
Northwestern University Medical School, Chicago

A. C. CORCORAN, M.D.
Cleveland Clinic, Cleveland

ELMER L. DeGOWIN, M.D.
State University of Iowa, Iowa City

EUGENE B. FERRIS, M.D.
Emory University School of Medicine, Atlanta

E. M. K. GEILING, M.D.
University of Chicago, Chicago

WENDELL H. HALL, M.D.
University of Minnesota, Minneapolis

THOMAS H. HUNTER, M.D.
University of Virginia, Charlottesville

LEON O. JACOBSON, M.D.
University of Chicago, Chicago

OLIVER H. LOWRY, M.D.
Washington University, St. Louis

RANDALL G. SPRAGUE, M.D.
Mayo Clinic, Rochester

RICHARD W. VILTER, M.D.
University of Cincinnati, Cincinnati

Vol. 44, No. 1, July, 1954. The Journal of Laboratory and Clinical Medicine is published monthly by The C. V. Mosby Company, 3207 Washington Blvd., St. Louis 3, Mo. Subscription price: United States and its Possessions $12.00 per year; Canada $12.50; Foreign $13.50. Subscriptions to medical students, interns and residents, half-price. Entered as Second Class Matter October 8, 1915, at the Post Office at St. Louis, Missouri, under Act of March 3, 1879. Printed in the U. S. A.

Medical
Wahr.

The Journal of Laboratory and Clinical Medicine

VOL. 44 JULY, 1954 No. 1

CLINICAL AND EXPERIMENTAL

OBSERVATIONS IN AIR FORCE RECRUITS OF STREPTOCOCCAL DISEASES AND THEIR CONTROL WITH ORALLY ADMINISTERED PENICILLIN

STANLEY H. BERNSTEIN, CAPTAIN, USAF (MC),* HARRY A. FELDMAN, M.D.,** O. F. HARPER, JR., CAPTAIN, USAF (MSC), W. H. KLINGENSMITH, FIRST LIEUTENANT, USAF (MSC), AND JEROME A. CANTOR, CAPTAIN, USAF (MC)
SYRACUSE, N. Y.

GROUP A streptococcal infections and their sequelae, rheumatic fever and acute glomerulonephritis, represent serious problems for the Armed Forces as well as for the civilian population. The former, consequently, long have been concerned with the development of a method that would be suitable as a mass preventive of such infections. The advent of the present era of antibacterial agents renewed interest in the search for a streptococcal prophylactic that would be effective against all Group A streptococci, simple to administer, and free of serious toxicity when prescribed for large numbers of men.

In 1942, Watson and associates[1] apparently were able to control a scarlet fever epidemic by employing sulfadiazine as a prophylactic agent. During World War II, the United States Navy conducted widespread field studies designed to evaluate the same drug as a mass prophylactic for streptococcal diseases. These experiences have been summarized by Coburn and Young,[2] in an extensive report in which it was concluded that sulfadiazine could be effectively utilized for this purpose. A serious limitation of the value of sulfadiazine became evident when streptococci resistant to the drug were detected in a number of locales.[3-9] These strains were of Types 17, 19, and, to a lesser extent, 3. Johnson and Hartman[10] later described, from a civilian community, an epidemic which was caused by sulfadiazine-resistant Type 19 streptococci.

These studies were conducted under the auspices of the Division of Preventive Medicine, United States Air Force, and with the support of the School of Aviation Medicine of the United States Air Force.

Received for publication Dec. 7, 1953.

*Project Officer, Epidemiological Detachment, assigned Sampson Air Force Base from Headquarters USAF.

**Associate Professor of Medicine, State University of New York, Upstate Medical Center at Syracuse, and Consultant USAF Hospital, Sampson Air Force Base. Supported by a grant from the Masonic Foundation for Medical Research and Human Welfare.

In 1945, Bynoe and his associates[11] conducted a study among members of the Royal Canadian Navy who had been hospitalized because of pharyngitis proved by culture to be caused by Group A streptococci. Penicillin was administered either intramuscularly or orally, and it was found that the exudative pharyngitis responded promptly, and that the positive throat cultures were reversed by either method of penicillin administration. These authors suggested that orally administered penicillin might be an excellent prophylactic agent for streptococcal infections.

Hamburger and Lemon[12] in the following year reported that 300,000 units of calcium penicillin in beeswax peanut oil, administered intramuscularly daily for five to seven days, eradicated hemolytic streptococci from the noses and throats of one-half of a small group of carriers. Streptococci were reduced in numbers or eliminated from the other half of this group, but their positive cultures recurred following the completion of the treatment period. It was demonstrated in the same study that 1 Gm. of sulfadiazine daily was quite effective in controlling streptococcus carriers unless there were sulfonamide-resistant streptococci present, in which case penicillin could be efficacious.

Limited field trials with penicillin as the sole prophylactic for streptococcal infections have been carried out among a number of military populations during the past several years. In the study reported by Wannamaker and associates,[13] it was found that oral doses of 500,000 or 1,000,000 units twice daily diminished the carrier and case rates provided that such treatment was maintained at least for ten days. Gastrointestinal disturbances were rather annoying with either of these dosages. The same authors reported also that 250,000 units, orally, once daily for ten days was inadequate for prophylaxis. In a study to be published in detail elsewhere[14] it was determined that 250,000 units twice daily was not only effective in lowering the carrier and case rates but was also relatively nontoxic and easy to administer.

In the present report a series of mass field trials in which the prophylactic value of orally-administered penicillin was evaluated in large numbers of Air Force Recruits when Group A streptococcal diseases were present with considerable frequency will be described. Additional information concerning certain epidemiologic aspects of streptococcal diseases in a basic military training center also will be presented.

The project was conducted under the auspices of the Department of Preventive Medicine of the Air Force and the School of Aviation Medicine among recruits stationed at the Sampson Air Force Base Recruit Training Center.

METHODS AND MATERIALS

The physical and geographical arrangements of recruit personnel within the troop training areas of Sampson Air Force Base were advantageous for investigations of a field trial nature. Recruits from the eastern and northeastern sectors of the United States were inducted into the Air Force at Sampson for twelve weeks of basic training prior to assignment elsewhere. Basic trainees, upon arrival, were formed into flights of 64 to 72 men which were allocated, alternately, to one of the two large troop areas (Fig. 1). Trainees were acquired by and lost from each area to approximately the same extent throughout the study period. Group A streptococcal pharyngitis and scarlet fever were present in high and practically equal incidence in both troop areas.

Commercial, buffered tablets containing 250,000 units of crystalline penicillin G were administered twice daily (one hour before or two hours after breakfast and again at bedtime) to each man in a test area. Placebos were not used in the control population. In the first prophylaxis trial, the penicillin was administered to all recruits in Troop II (each troop contained approximately one-half of the recruits on the base) for a ten-day period while in the second, the same troop received the drug for five days. In the third trial, one-third of Troop I (D Area) was given penicillin for five days. Finally, the entire training base population received ten days of prophylactic penicillin treatment.

The penicillin tablets were distributed by the flight instructors who had received appropriate indoctrination. Each recruit was questioned concerning previous penicillin sensitivity reactions and, if he had had such, he was not given the drug. Any man who developed a suggestive penicillin reaction was referred immediately to the Dermatology Section of the base hospital for further evaluation and treatment.

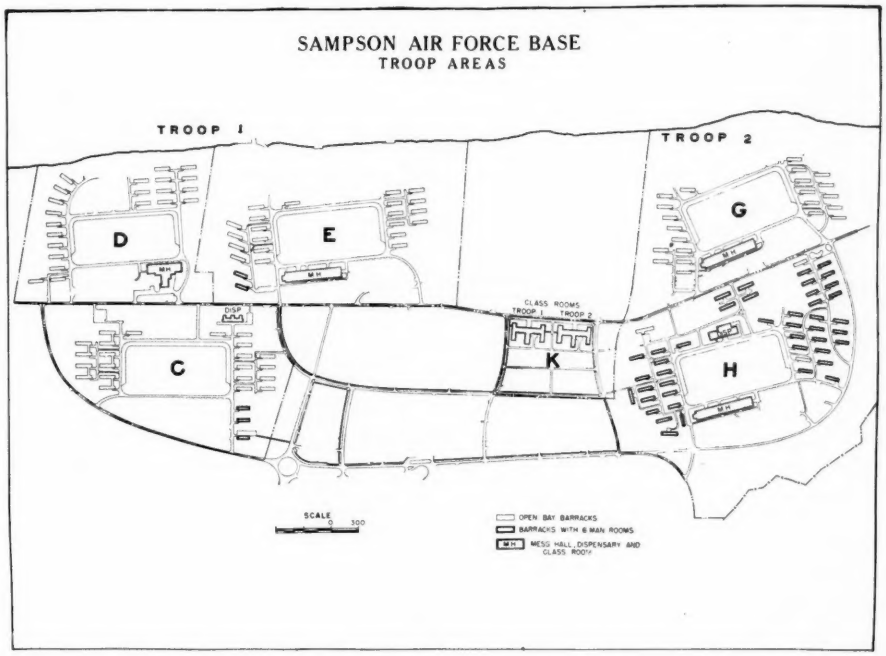

Fig. 1.—Troop area map of the Sampson Air Force Base.

Beginning on Nov. 1, 1952, recruits who came to base dispensaries or sick call with oral temperatures of 100° F., or greater, and with symptoms of upper respiratory or pulmonary infections, were referred to the hospital for admission. Antimicrobial therapy was withheld until the patients arrived at their hospital beds, at which time their pharynges were swabbed; sheep's blood agar plates were inoculated from these swabs within thirty minutes.

A diagnosis of streptococcal disease was made if the patient had either sarlet fever, with or without a positive throat culture or exudative pharyngitis associated with a throat culture that was positive for Group A streptococci. The pharynx of each patient who had a positive throat culture was examined for exudate by one of two medical officers who were members of this unit. These criteria were rather stringent and it is not improbable that the streptococcal disease rates would have been even higher had other standards been used.

Pharyngeal streptococcal carrier surveys (on approximately 15 per cent samples) were performed among the entire recruit population at weekly intervals during each prophylaxis trial.

Streptococcal typing* was carried out[16] on a large sample of the strains which had been obtained from hospitalized cases and ambulant carriers, as well as with all streptococci isolated from patients with either rheumatic fever or acute glomerulonephritis.

RESULTS

Mass Oral Penicillin Prophylaxis Administered for a Ten-Day Period.—Streptococcal disease rates, as determined from hospital admissions, reached epidemic proportions during January, 1953 (Fig. 2). During the week of

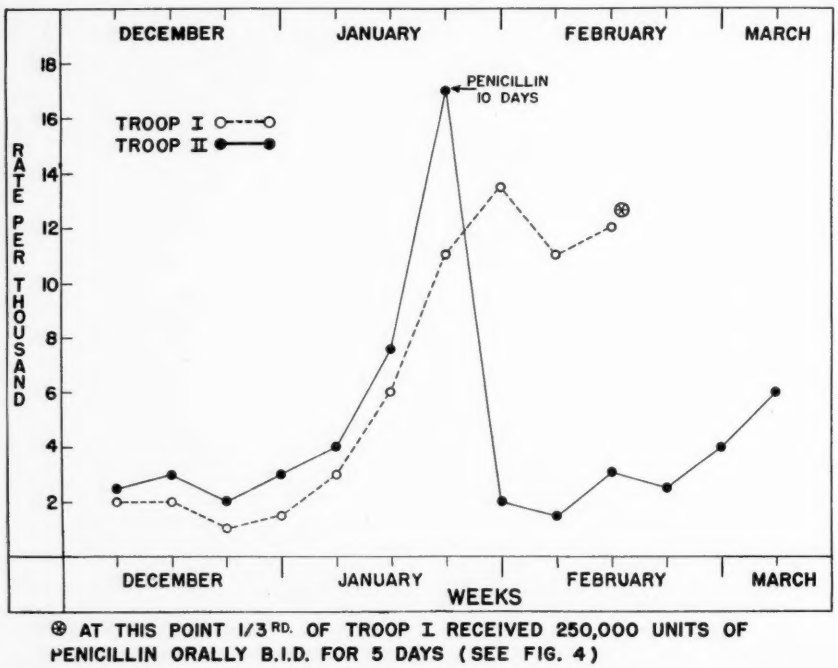

Fig. 2.—Effect of ten days of orally administered penicillin (250,000 units b.i.d.) upon the rate of streptococcal disease in a recruit population.†

January 25, when the rates were 17 per thousand per week in Troop II and 13 per thousand per week in Troop I, penicillin was administered for ten days to all recruits and the permanent part of Troop II. New flights assigned to Troop II during the ten-day prophylaxis period received similar treatment. Hospital admissions for streptococcal disease declined sharply within forty-eight hours after the penicillin was begun with the rates being lowered to 2 per thousand per week in the first week and 1.5 per thousand per week in the following week. Although the streptococcal disease rate remained high in Troop I (control), that of Troop II remained low for the five to six weeks following the treatment period.

Mass Oral Penicillin Prophylaxis Administered for a Five-Day Period.—Since the streptococcal disease hospital admission rates were again at signifi-

*The streptococcal grouping and typing sera were obtained from Dr. Elaine Updyke, Communicable Diseases Center, Department of Public Health, Education and Welfare, Chamblee, Ga. Antisera for Types 1, 2, 3, 4, 5, 6, 11, 12, 13, 14, 15, 17, 18, 19, 22, 23, 24, 26, 27, 28, 29, 30, 31, 32, 33, 36, 37, 38, 39, 40, 41, 42, 43, 44, 46, 47 were available for this project.

†Data in Figs. 2, 5, and 7 also appeared in an article by Bernstein, Feldman, Harper, and Klingensmith, in the June, 1954, A.M.A. Arch. Int. Med.

cant levels in both troops in the first week of March, 1953, another trial was initiated in which all Troop II recruits received the penicillin for five days (Fig. 3). There was an immediate, sharp decrease in disease incidence in this troop which was maintained, essentially, only during the period of penicillin administration. Three weeks after the cessation of prophylaxis, Troop II again had an elevated streptococcal disease rate.

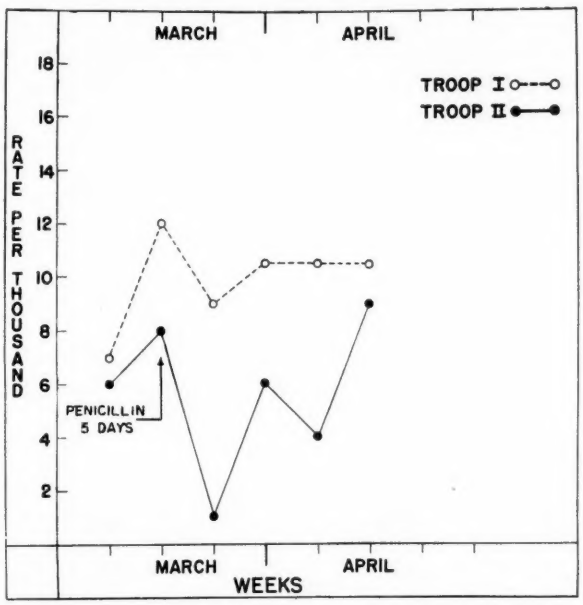

Fig. 3.—Effect of five days of orally administered penicillin (250,000 units b.i.d.) upon the rate of streptococcal disease in a recruit population.

Mass Oral Penicillin Prophylaxis Administered to One-Third of Troop I for a Five-Day Period.—During February, 1953, penicillin was administered to one-third of Troop I in order to determine the extent to which treatment of part of a troop would affect the streptococcal disease rate of the entire troop. Recruits assigned to Area A of Troop I received the drug and there was an immediate decrease in streptococcal infections. Members of Area C (untreated) also had a decrease in disease rate, but their counterpart, Area E, maintained a high disease rate despite the intermingling of men from all three areas. With the conclusion of the five-day treatment period, the rates promptly returned to epidemic levels (Fig. 4).

Mass Oral Penicillin Prophylaxis Administered to an Entire Trainee Population for Ten Days.—In the third week of April, 1953, streptococcal disease rates again being epidemic in both troops, oral penicillin prophylaxis was instituted for ten days among all members of the base trainee population (Fig. 5). The result was similar to that previously observed during the similar trial in Troop II, in that the disease rate promptly declined to less than one per thousand per week for all recruits.

In order to gain additional information, all new flights subsequently assigned to Troop II were treated for ten days with the same dose of penicillin,

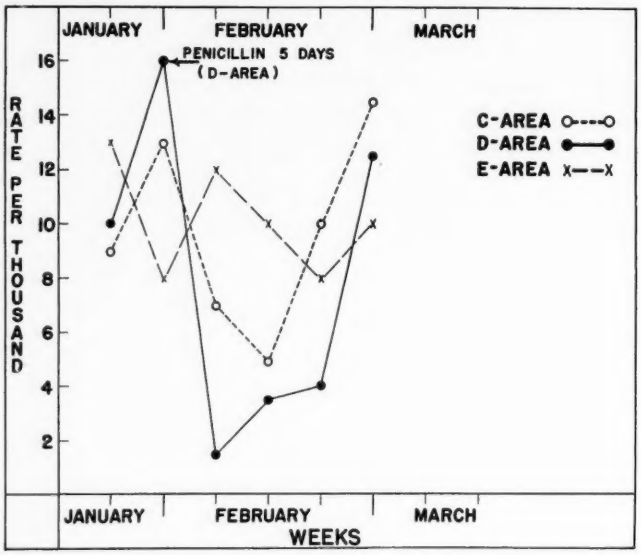

Fig. 4.—Effect upon the streptococcal disease rates of administering penicilliin (250,000 units b.i.d., orally) to one-third of a troop for five days.

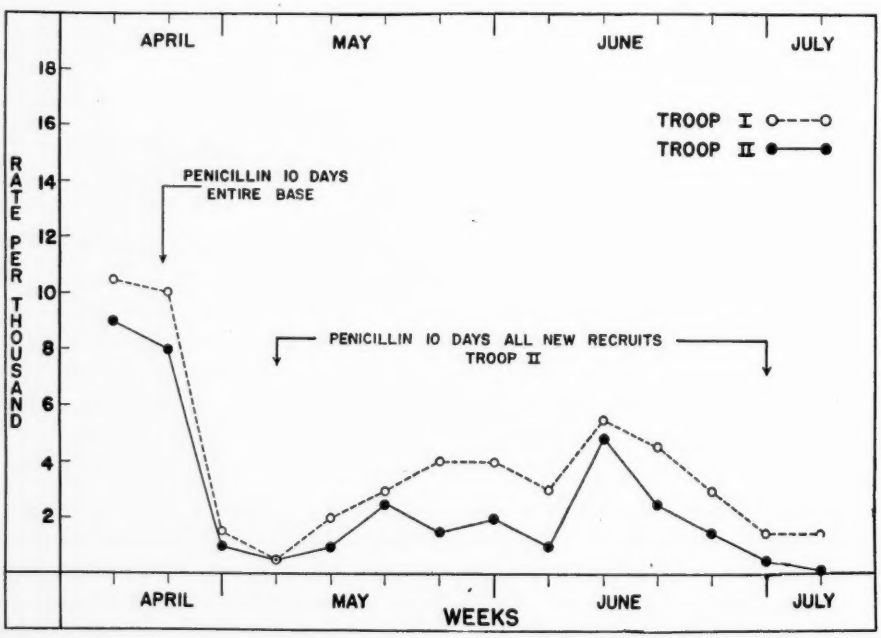

Fig 5.—Effect upon streptococcal disease rates of administering penicillin orally (250,000 units b.i.d.) for ten days to the entire base population followed by similar treatment of each new recruit entering one troop area.

while those entering Troop I were not. Somewhat inconclusive results were noted in this portion of the study (Fig. 5), for the weekly streptococcal disease rates of Troop II, during the period of administration of the drug to incoming recruits, were lower than those of Troop I, but a statistically significant difference was not noted in each week. The sharp increase in rate (Fig. 5) noted during the fifth week after this program had been initiated probably represents hospital admissions for streptococcal diseases among men who had received prophylaxis five weeks previously. No admissions were noted from among the new men undergoing prophylaxis. This type of study should be repeated at a time when the incidence of streptococcal diseases is greater so that this system of prophylaxis may be evaluated with greater certainty.

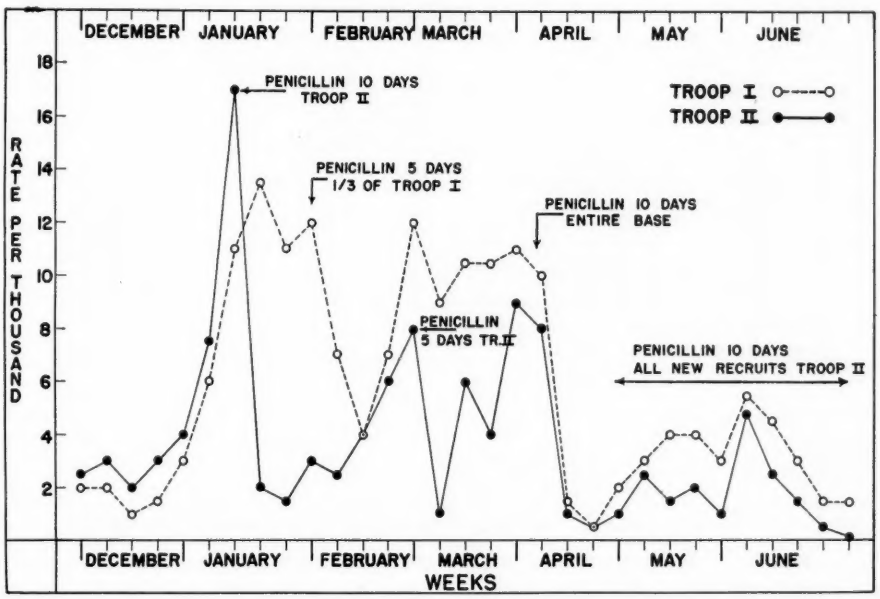

Fig. 6.—Effects upon streptococcal disease rates of penicillin orally administered (250,000 units b.i.d.) for varying lengths of time to Air Force recruit populations.

All of the penicillin prophylaxis studies described previously are summarized compositely in Fig. 6. The total numbers of hospital admissions for streptococcal diseases among the recruit populations studied were as follows: Troop I, 1,188 cases, Troop II, 667 cases; 700 (37 per cent) of these 1,855 cases were diagnosed as having scarlet fever. The various trials apparently prevented about 500 cases of streptococcal disease in Troop II. Since the usual patient spends five days in the hospital, approximately 2,500 man hospital days were prevented during the first three trials. The effectiveness of the last trial in this regard cannot be calculated, but it evidently was not inconsiderable.

As mentioned previously, carrier surveys (Fig. 7) were carried out during the various study periods. Both the hospital admissions rates for streptococcal diseases and the percentages of positive cultures obtained from the throats of apparently healthy trainees were affected similarly by the various penicillin regimens.

Streptococcal Types.—Tables I and II show the distribution of Group A streptococcal types which were isolated from the hospitalized cases and during the carrier surveys. It is apparent that Type 3 was predominant in both instances and that Types 12, 42, and 28 were the most frequent subordinate types. Despite the several mass penicillin trials, Type 3 maintained its dominant position throughout the season, which might indicate that recruits who enter a military installation during an epidemic streptococcal period acquire the streptococcus which is predominant in the camp, rather than introducing new types.

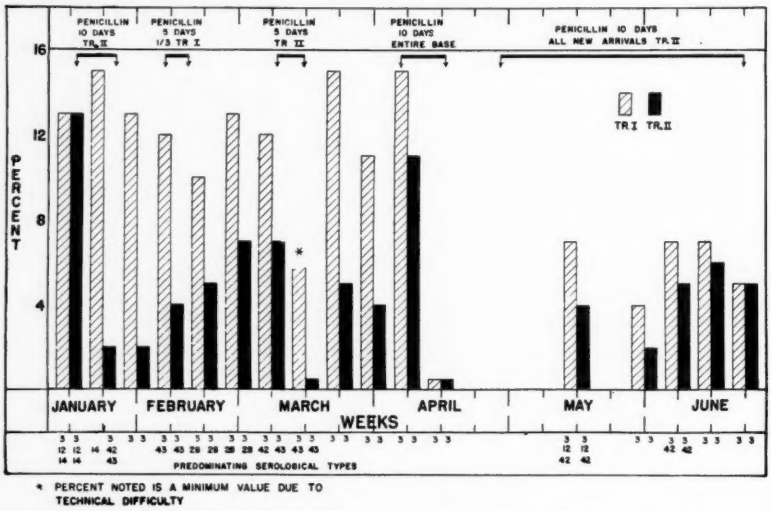

Fig. 7.—Effects of various oral penicillin regimens (250,000 units b.i.d.) upon Group A streptococcal carrier rates.

Sensitivity Reactions to Orally Administered Penicillin.—The number of individuals with histories of previous penicillin sensitivity reactions was remarkably similar to the number of recruits who manifested such reactions during the various prophylaxis trials. Although this information will be reported in detail elsewhere,[17] some of the data are summarized in Table III. Of the 33,827 men who received penicillin prophylaxis, 90 (0.3 per cent) had reactions. Urticaria occurred in 43 of these 90 men and 19 had a serum-sickness-like disease. Among the latter, eight required ACTH or cortisone for relief of symptoms. It is interesting that Gezon and associates[18] encountered a similar incidence of sensitivity reactions to penicillin orally administered in dosage of 125,000 units daily to Naval recruits. These authors gave the penicillin for about one month and it is not evident whether this smaller dose would have resulted in fewer reactions had the period of exposure been only ten days. Consequently, it is not possible to state whether, in short courses, the larger dose reported herein is any more likely to produce sensitivity reactions.

Diarrhea, which was reported as a toxic reaction by Wannamaker and associates[13] following daily oral doses of one to two million units, was not en-

TABLE I. DISTRIBUTION OF STREPTOCOCCAL TYPES AMONG HOSPITALIZED CASES OF STREPTOCOCCAL PHARYNGITIS

TYPE	TROOP I		TROOP II		TOTAL	
	NO.	PER CENT	NO.	PER CENT	NO.	PER CENT
1	7	1.3	2	1.0	9	1.2
2	1	0.2	0	0.0	1	0.1
3	347	66.2	135	68.5	482	66.9
4	1	0.2	1	0.5	2	0.3
12	36	6.9	7	3.6	43	6.0
14	23	4.4	3	1.5	26	3.6
17	2	0.4	0	0.0	2	0.3
19	2	0.4	3	1.5	5	0.7
24	8	1.5	2	1.0	10	1.4
26	3	0.6	0	0.0	3	0.4
28	27	5.2	11	5.6	38	5.3
29	0	0.0	1	0.5	1	0.1
36	6	1.1	3	1.5	9	1.2
37	0	0.0	1	0.5	1	0.1
39	1	0.2	0	0.0	1	0.1
40	5	1.0	0	0.0	5	0.7
41	11	2.1	6	3.0	17	2.4
42	30	5.7	12	6.1	42	5.8
43	12	2.3	7	3.6	19	2.6
44	1	0.2	0	0.0	1	0.1
Nontypable	1	0.2	3	1.5	4	0.6
Total	524	100	197	100	721	100

TABLE II. COMPARATIVE INCIDENCES OF STREPTOCOCCAL TYPES AMONG CARRIERS AND HOSPITALIZED CASES

TYPE	HOSPITALIZED		CARRIERS	
	NO.	PER CENT	NO.	PER CENT
1	9	1.2	1	0.2
2	1	0.1	0	0.0
3	482	66.9	313	58.0
4	2	0.3	0	0.0
12	43	6.0	31	5.7
14	26	3.6	24	4.4
17	2	0.3	0	0.0
19	5	0.7	0	0.0
23	0	0.0	1	0.2
24	10	1.4	2	0.4
26	3	0.4	0	0.0
28	38	5.3	62	11.5
29	1	0.1	0	0.0
30	0	0.0	1	0.2
36	9	1.2	1	0.2
37	1	0.1	0	0.0
39	1	0.1	0	0.0
40	5	0.7	1	0.2
41	17	2.4	0	0.0
42	42	5.8	53	9.8
43	19	2.6	41	7.6
44	1	0.1	0	0.0
47	0	0.0	1	0.2
Nontypable	4	0.6	8	1.5
Total	721	100	540	100

countered with the daily 500,000 units employed by us. As might have been expected in a study which was conducted during cool weather, there were no obvious exacerbations of epidermatophytosis.

All trainees who were hospitalized because of streptococcal diseases and who had not received prophylaxis during the epidemic period, January to March, 1953, were investigated as to how long they had been in training (Fig. 8) before their first symptoms of streptococcal illness appeared. The majority of men became ill between the second and fifth weeks of training, which suggests that recruits could be carried through the ten- to twelve-week training period without major streptococcal difficulties if penicillin prophylaxis were provided both in the second and fifth weeks.

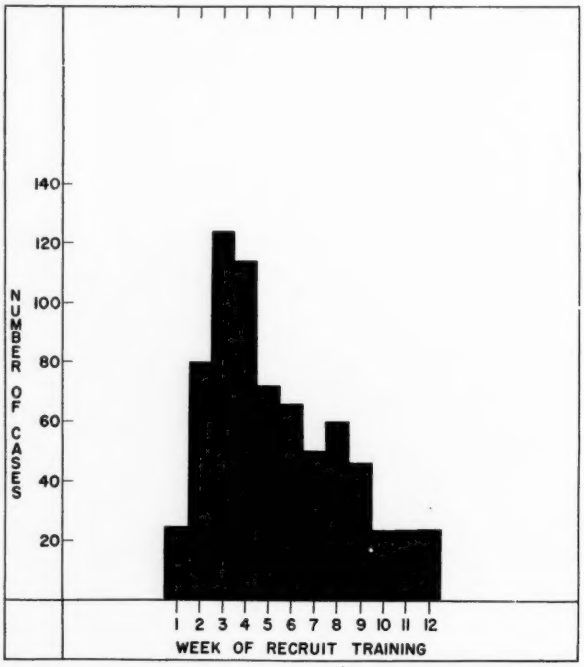

Fig. 8.—Time of onset of Group A streptococcal pharyngitis in relation to the week of training.

Nine cases of acute glomerulonephritis were detected during the season in which the present prophylaxis studies were conducted. As is shown in Table IV, six of these patients had throat cultures positive for Type 12 streptococci when admitted to the hospital. This is especially interesting since Type 3 occurred eleven times more often than Type 12 during this season, which supports the observations of Rammelkamp and Weaver[19] and Wertheim and his colleagues[20] that Type 12 is particularly prone to precipitate acute glomerulonephritis.

DISCUSSION

From the data which have been presented, it appears that crystalline, buffered penicillin G, administered orally in a dosage of 250,000 units twice daily, is an effective mass prophylactic against the Group A streptococcus provided

TABLE III. INCIDENCE OF SENSITIVITY REACTIONS TO ORAL PENICILLIN COMPARED WITH THE INCIDENCE (BY HISTORY) OF PREVIOUS REACTIONS IN THE SAME POPULATION

TRIAL	DAYS OF TREATMENT*	PENICILLIN REACTIONS	HISTORY OF PREVIOUS PENICILLIN REACTION (EXCUSED FROM STUDY)
		PER CENT	PER CENT
1	10	0.60	0.70
2	5	0.30	0.30
3	5	0.10	0.20
4	10	0.20	0.40

*250,000 units b.i.d.

Of the 33,827 men who received oral penicillin in these trials, 90 (0.3 per cent) had sensitivity reactions and 124 (0.4 per cent) were not given the drug because of histories of previous sensitivity reactions.

TABLE IV. PREVALENCE OF TYPE 12 STREPTOCOCCI IN THROAT CULTURES OF PATIENTS WITH ACUTE GLOMERULONEPHRITIS

PATIENT	ADMISSION CULTURE	STREPT. TYPE
J. W.	Positive	12
W. S.	Positive	12
R. K.	Positive	12
W. D.	Positive	12
S. G.	Positive	12
A. P.	Positive	12
R. H.	Negative	—
E. S.	Negative	—
J. P.	Negative	—

that such treatment is continued for ten days. It is interesting that this prolonged period of treatment is necessary even when the daily penicillin dose is doubled or quadrupled as was reported by Wannamaker and associates.[13] The ten-day schedule not only stopped the streptococcal disease epidemic, but also reduced the incidence of Group A streptococcal pharyngeal carriers markedly for about six weeks, while five days of such penicillin treatment had a suppressive effect for only two to three weeks. The treatment of one-third of a large population reduced the over-all incidence of streptococcal disease in the entire population, but the end result was poor. It is possible that if this treatment period had been ten days in length, the effect would have been more noteworthy. When prophylaxis was administered to the total base personnel, it was very effective in aborting a streptococcal disease epidemic, but this trial was conducted too late in the season to determine the duration of the effect of such base-wide treatment.

The streptococcal types responsible for disease remained constant among hospitalized cases of streptococcal pharyngitis, which seems to support the thesis that under the conditions of these trials, the strains responsible for the epidemics were suppressed but not eliminated by the penicillin prophylaxis. Since the immediate result of each successive exhibition of penicillin was similar, it appears unlikely that the sensitivity to penicillin of these strains was altered.

The last study, in which all new recruits entering one troop area received the same prophylaxis which the entire base had already received, is of special interest. Although the epidemic year had probably ended, the carrier rates were apparently similar in both troops during this period, but a definite trend toward a lower rate of disease was noted in the troop whose incoming recruits

were being treated. This particular trial parallels a previous study by one of us (H. A. F.) in which a meningococcal meningitis epidemic was controlled by first administering sulfadiazine to the total military population and then providing similar treatment for all new inductees entering the area.[15] The meningococcal problem, however, is somewhat simpler in that epidemics are caused by only one serologic type which is readily eradicated by sulfonamides. Once the organism has been eliminated from a given population, then the problem becomes one of preventing its reintroduction by new recruits. In our experiences, as reported herein, it is apparent that even after ten days of penicillin, the streptococci responsible for the epidemic had not been totally eliminated and, consequently, they remained as a threat to the new recruits, which is the reverse of the meningococcal situation.

There were twenty cases of acute rheumatic fever admitted to this Base Hospital during the study period; all were recruit trainees. Seven of these had had previous admissions for respiratory infections. Three of the seven had had a Type 3 streptococcus isolated at the time of the previous respiratory infection. Eight of the twenty had histories of childhood rheumatic fever. It would appear that men who are inducted into the Armed Forces with histories of previous rheumatic fever might well be maintained on continuous penicillin prophylaxis, at least when serving at bases with high endemic streptococcal disease rates.

SUMMARY AND CONCLUSIONS

Mass prophylaxis with oral penicillin in dosage of 250,000 units twice daily for ten days suppressed hospital admissions for streptococcal disease markedly for as long as six weeks. Similar treatment for a five-day period was noticeably less effective. Similar effects were noted in carrier surveys of the associated well recruit populations.

Serologic types isolated from both hospitalized cases and carriers revealed a predominance of Type 3 streptococci throughout the study period; Types 12, 42, and 28 were present to a lesser extent.

Sensitivity reactions from the oral penicillin were infrequent (0.3 per cent) and mild.

Type 12 streptococci were isolated from the six of nine cases of acute glomerulonephritis who had positive throat cultures.

Recruits were prone to acquire streptococcal infections during the second and fifth weeks of a ten- to twelve-week basic training period.

The cooperation and support of Major General Richard C. Lindsay, Base Commander; Lt. Col. J. R. Patterson, Group Commander; Major W. T. Downey and Major W. R. Campbell, Jr., Troop Commanders, and the personnel of the 3650th Military Training Wing are acknowledged gratefully. We are indebted to Col. J. R. Copenhaver, Commandant, Base Hospital, Col. J. Tobin, Chief, Medical Service, Col. C. Z. Berry, Chief, Professional Services, and the personnel of the USAF Hospital, Sampson AFB, for their aid. It is a pleasure to acknowledge the organizational help and constructive criticism of Col. Fratis L. Duff, USAF (MC), Chief, Division of Preventive Medicine, USAF. We wish also to thank Lt. Col. H. V. Ellingson for his aid, and the USAF School of Aviation Medicine for its financial support.

We are indebted, also, to The Armed Forces Epidemiological Board for certain information which was of aid in the planning of this study.

Finally, an expression of our deepest appreciation to: T/Sgt. R. Stafford, T/Sgt. J. Briley, T/Sgt. T. Remson, T/Sgt. M. Coogan, S/Sgt. P. Lange, S/Sgt. C. Cummings, and Airmen Nelson, Bell, Vining, Moerschell, Cappetta, Simonds, Madore, Gentsch, and Sikes, members of the Epidemiological Detachment, 1141st Medical Services Squadron USAF.

REFERENCES

1. Watson, R. F., Schwentker, F. F., Fetherston, J. E., and Rothbard, S.: Sulfadiazine Prophylaxis in an Epidemic of Scarlet Fever, J. A. M. A. **122:** 730-733, 1943.
2. Coburn, A. F., and Young, D. C.: The Epidemiology of Hemolytic Streptococcus, Baltimore, 1949, Williams & Wilkins Company.
3. Epidemiology Unit No. 22, Sulfadiazine Resistant Strains of Beta Hemolytic Streptococci. Appearance During the Course of Sulfadiazine Prophylaxis at a Large Naval Training Center, J. A. M. A. **129:** 921-927, 1946.
4. Damrosch, D. S.: Chemoprophylaxis and Sulfonamide Resistant Streptococci, J. A. M. A. **130:** 124-128, 1946.
5. Van Ravenswaay, A. C., Westwater, J. A., and Holbrook, W. P.: An Epidemic Caused by a Sulfadiazine Resistant Strain of Group A Type 17 Streptococcus, M. Clin. North America **30:** 707-715, 1946.
6. Delamater, E. D., Jennings, R., and Wallace, A. W.: Preliminary Report of an Outbreak of Streptococcal Disease Caused by a Sulfadiazine Resistant Group A Type 17 Hemolytic Streptococcus, J. Infect. Dis. **78:** 118-127, 1946.
7. Wilson, O. G.: An Outbreak of Sulfadiazine Resistant Streptococcus Infection at Lowry Field, Colorado, J. Infect. Dis. **78:** 147-152, 1946.
8. Mitchell, R. B., Tuttle, E. E., Dingledine, L. C., Grams, L. R., Erdman, G. L., Lombs, F. S., and Holbrook, W. P.: The Inter-post Dissemination of Epidemic Strains of Hemolytic Streptococci by Troop Movement, J. Infect. Dis. **78:** 134-218, 1946.
9. Roberg, N. B.: An Epidemic Caused by a Sulfadiazine Resistant Strain of the Streptococcus Hemolyticus (Group A (Type 17), J. Infect. Dis. **78:** 135-146, 1946.
10. Johnson, R. D., and Hartman, T. L.: Sulfadiazine Resistant Streptococcal Infections in a Civilian Community, J. Clin. Invest. **26:** 325-328, 1947.
11. Bynoe, E. T., MacLennan, J., Williamson, J., Carpenter, J., and Armstrong, C.: Penicillin in Hemolytic Streptococcal Infections of the Throat, Canad. M. A. J. **53:** 471-478, 1945.
12. Hamburger, M., Jr., and Lemon, H. M.: The Problem of the Dangerous Carrier of Hemolytic Streptococci. III. The Chemotherapeutic Control of Nasal Carriers, J. A. M. A. **130:** 836-841, 1946.
13. Wannamaker, L. W., Denny, F. W., Perry, W. Y., Rammelkamp, C. H., Jr., Eckhardt, G. C., Houser, H. B., and Hahn, E. O.: The Effect of Penicillin Prophylaxis on Streptococcal Disease Rates and the Carrier State, New England J. Med. **249:** 1-7, 1953.
14. Bernstein, S. H., Feldman, H. A., Harper, O. F., Jr., and Klingensmith, W. H.: Mass Oral Penicillin Prophylaxis in the Control of Streptococcal Disease, Arch. Int. Med. In press.
15. Feldman, H. A.: Unpublished observations, 1943.
16. Swift, H. F., Wilson, A. T., and Lancefield, R. C.: Typing Group A Hemolytic Streptococci by M Precipitin Reactions in Capillary Pipettes, J. Exper. Med. **78:** 127-133, 1943.
17. Berry, C. Z., and Ferber, J.: Reactions to Penicillin Administered Orally for Mass Prophylaxis of Streptococcal Disease. To be published.
18. Gezon, H. M., Cook, J. S., Jr., Magoffin, R. L., and Miller, C. H.: The Use of Penicillin and Sulfadiazine as Prophylactic Agents Against Streptococcal and Non-specific Respiratory Infections Among Recruits at a Naval Training Center, Am. J. Hyg. **57:** 71-100, 1953.
19. Rammelkamp, C. H., Jr., and Weaver, R. S.: Acute Glomerulonephritis. The Significance of the Variations in the Incidence of the Disease, J. Clin. Invest. **32:** 345-355, 1953.
20. Wertheim, A., Lyttle, J. D., Loeb, E. N., Earle, D. P., Jr., Seegal, B. C., and Seegal, D.: The Association of Type Specific Hemolytic Streptococci With Acute Glomerulonephritis at the Presbyterian and Babies Hospitals, New York, N. Y., 1936-1942, J. Clin. Invest. **32:** 359-363, 1953.

BACTERIOPHAGE TYPING OF STAPHYLOCOCCI

I. Technique and Patterns of Lysis

George Gee Jackson, M.D., Harry F. Dowling, M.D., and
Mark H. Lepper, M.D.
Chicago, Ill.

ADVANCEMENT in the investigation of clinical infections with *Micrococcus pyogenes* has been seriously impeded by the lack of a satisfactory method of identifying specific strains. The ubiquitous distribution of the species and the high carrier rate among well persons have exaggerated the disadvantage of being unable to type staphylococci. A few tests based upon hemolysis, pigment, and coagulase production have served to distinguish broad groups among the species and to indicate virulence or potential virulence of the strain, but they have little information as to the identity of individual strains.

Within the past few years, methods of identifying staphylococci according to their susceptibility to groups of bacteriophages have been developed by Burnet and Lush,[1] Williams and Timmins,[2] Fisk,[3-5] Wilson and Atkinson,[6] and others, and are beginning to be employed extensively in a number of laboratories, especially in England, Australia, and Canada.

Bacteriophage typing of staphylococci was begun in our laboratory in 1951 as an aid in the epidemiologic observations upon infections of the respiratory tract and in the study of antibiotic resistant strains of staphylococci. In the present publication, an analysis of the results obtained in testing 2,085 strains of staphylococci isolated from 795 persons by means of twenty phages will be presented, along with the technique employed, and the patterns observed, among the phages which were used.

MATERIALS AND METHODS

Isolation of the Bacterial Strains.—The principal portion of the study was conducted on the wards of the Chicago Municipal Contagious Disease Hospital. Nose and throat cultures were obtained at the time of hospital admission from every second, third, or fourth patient, depending on the rate of admissions and regardless of the clinical diagnosis. Strains of *M. pyogenes* were isolated in pure culture from a streak-plate containing 6 per cent human blood agar. Subsequent cultures were obtained from the patients approximately two times a week until they were discharged from the hospital. All of the household contacts of the patient who would permit the taking of cultures were studied prior to the release of the patient. Additional cultures were obtained from both the patient and his household contacts, two, six, and ten to twelve weeks after the patient had been discharged from the hospital, when this was possible.[7] No cultures were taken from household contacts or patients beyond discharge from the hospital after May, 1952. During the entire period of the study, a large majority of the hospital personnel, comprising approximately one hundred persons, had nose and throat cultures taken at monthly intervals.

From the Department of Medicine, Research and Educational Hospitals, and Chicago Municipal Contagious Disease Hospital, University of Illinois College of Medicine, Chicago, Ill.
Received for publication April 1, 1954.

Additional strains of *M. pyogenes* were obtained from other patients with suppurative illnesses of staphylococcic etiology, as well as from patients with similar illnesses at the University of Illinois Research and Educational Hospitals.*

In 1953, thirty-nine strains of *M. pyogenes* isolated from the stools of patients at the Mayo Clinic with staphylococcic enteritis were furnished by Dr. W. H. Heilman, and, in addition, thirty-five strains isolated concomitantly from sources other than the colon of patients at the same institution.

Dr. Maxwell Finland provided a similar group of thirty-five strains isolated from the stools and seventy-five strains isolated from the respiratory tract of patients at the Boston City Hospital.

Fifteen antibiotic-resistant strains of *M. pyogenes* were supplied by Dr. Ernest Jawetz from patients at the University of California Hospital.

Coagulase tests were performed by the method of Fisk.[8] The substrate used was a 1:10 dilution of fresh rabbit plasma.

Alpha hemolysin tests were performed by the method of MacFarlan.[9] A titer of 1:10 or greater was considered positive, and a lower titer was considered doubtful.

The Identity of the Phages.—Strains of staphylococci often carry a bacteriophage which is not lysogenic for the carrier-strain.[3] A technique for the isolation of these bacteriophages has been given in detail in the publications of Fisk.[3-5] A large number of such phages have been isolated and propagated by the British workers who have been prominent in the advancement of this technique.[10] The phages have been classified by these investigators, according to their reaction patterns, into three broad groups plus certain unclassified phages.[11] The identity of the phages by the laboratory numbers is shown by the grid in Table I.† Three Group I phages which were used were *29, 31,* and *52.* Three Group II phages are designated *3A, 3C,* and *51.* Eight phages were used from Broad Group III. They were *6, 7, 42B, 47, 47B, 47C, 53,* and *54.* The miscellaneous phages were *W, 977, 1272, 1791, 971,* and *47A.* Among the latter group, the first four consist of the W phage, which was isolated by Colbeck[12] from an epidemic of staphylococcic infections among infants, and three strains adapted from the W phage. It is apparent that they have great similarity, if, indeed, they are not identical.

Propagation of the Phages.—Each of the phages has been propagated by means of a standard phage-susceptible strain of *M. pyogenes.* The propagating strain and phage have been paired in Table I. A semisolid agar medium was used for propagation. Among a number of modifications of the method which were tried, the agar layer method described by Swanstrom and Adams[13] was found most satisfactory.

The base layer of medium which was poured into the Petri plate was composed of 1.5 per cent nutrient agar adjusted to a pH of 8.0. The plate was incubated overnight at 37° C. to dry the agar and to assure sterility. The surface layer was prepared by placing 0.1 ml. of a four-hour nutrient broth culture of the propagating strain into 2.5 ml. of molten 0.8 per cent nutrient agar adjusted to pH 8.0, and adding 0.1 ml. of stock phage. The tube was gently mixed and poured over the base agar plate. After sixteen to eighteen hours' incubation at 30° C., the plate was flooded with 5 ml. of nutrient broth and the surface agar scraped free with the tip of a pipette. The harvests from several Petri plates were transferred to a test tube and centrifuged at high speed. The supernate was aspirated and the phage titer determined by testing serial log-dilutions against the propagating strain. A typical titration is shown in Fig. 1. The greatest dilution which produced complete confluent lysis, 10^{-7} in the figure shown, was designated the critical test dose (C. T. D.). For most of the phages used, the C. T. D. was observed at 10^{-4} to 10^{-6} dilution. When the initial propagation yielded low phage titers, serial propagation was performed, the harvest from the preceding generation being used each time for the infecting inoculum. When a satisfactory titer was obtained, the

*The cooperation of Mary Turner, Elizabeth Pond, and Dorothy Powers of the Clinical Bacteriology Laboratory in the isolation of cultures is gratefully acknowledged.

†The phages used in the observations to be reported were kindly furnished with their propagating strains by Dr. G. B. Leyton, former Director of Laboratory Services, Bacteriological Laboratory, Department of Health and Public Welfare, Winnipeg, Manitoba, Canada.

TABLE I. LYSOGENIC PATTERNS OF STAPHYLOCOCCIC BACTERIOPHAGES AND THEIR PROGATING STRAINS

PROPAGATING STRAIN NO.	USED FOR PHAGE	PHAGES																				
		GROUP I			GROUP II			GROUP III								MISCELLANEOUS						
		29	31	52	3A	3C	51	6	7	42B	47	47B	47C	53	54	47A	W	977	1272	1791	971
33	29	*4+*																			
18	31		*4+*																		
144	52	1+	1+	*4+*						1+											
284	3A				*4+*	4+	4+														
1,339	3C				3+	*4+*															
145	51				4+	4+	*4+*														
3	6							*4+*	4+					4+	3+						4+
4	7								*4+*					4+							
1,163	42B									*4+*			4+								
36	47								3+		*4+*			3+							
987	47B											*4+*									
1,163	47C									4+			*4+*								
R48/3,292	53								4+					*4+*							
R48/R3,303	54								4+		4+		1+	4+	*4+*						
761	47A															*4+*					
8	W																*4+*	4+	4+	4+	
977	977																4+	*4+*	4+	4+	
1,272	1,272																4+	4+	*4+*	4+	
1,791	1,791																4+	4+	4+	*4+*	
971	971							4+	4+	4+				4+	4+		1-				*4+*

supernate was centrifuged until clear or filtered through a sintered glass filter and stored in stoppered vials at 4° C. Under these conditions, the phages showed no appreciable decrease in the titer after they had been stored for four to six weeks. Some phages have retained their titers after storage for three to six months. When the titer of the stock phage decreased more than one tenfold dilution, the phage was repropagated.

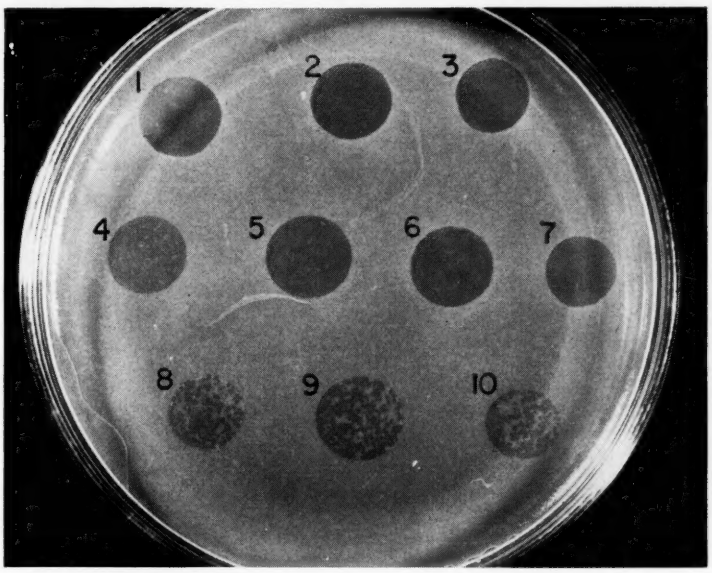

Fig. 1.—Titration of phage by means of serial tenfold dilutions. (Numbers indicate the reciprocals of the log-dilutions.) The critical test dose (C.T.D.) is 10^{-7}, i.e., the highest dilution which will produce confluent lysis of a standard strain.

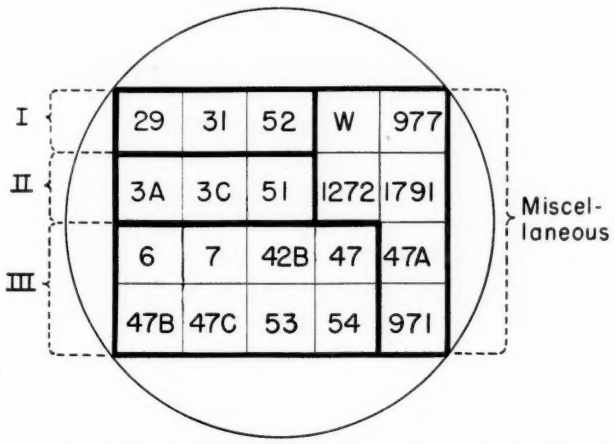

Fig. 2.—Format for bacteriophage testing of staphylococci. (The heavy lines separating the groups do not appear on the outline which is stamped on the Petri plate.)

Typing of Unknown Strains.—In the phage typing of unknown strains of *M. pyogenes*, a concentration of phage ten times greater than the critical test dose was used. This was designated the routine test dose (R. T. D.). The technique was adapted from that of Williams and Rippon.[11] Petri plates were prepared with the test medium, which consisted of 0.9 per cent nutrient agar adjusted to pH 8.0. The plates were then incubated overnight at 37° C. One milliliter of an overnight culture of the unknown strain in nutrient broth or tryptose

phosphate broth was flooded over the surface of the plate and the excess fluid aspirated. The plates were then placed back in the incubator with lids ajar for thirty minutes. The bottom of each plate had previously been marked with an indelible stamp which divided the plate into twenty squares, as illustrated in Fig. 2. When the surface of the plate was sufficiently dry, one drop of R. T. D. of each phage was delivered to the appropriate area from a 27-gauge hypodermic needle attached to a tuberculin syringe held in a horizontal position. Care was

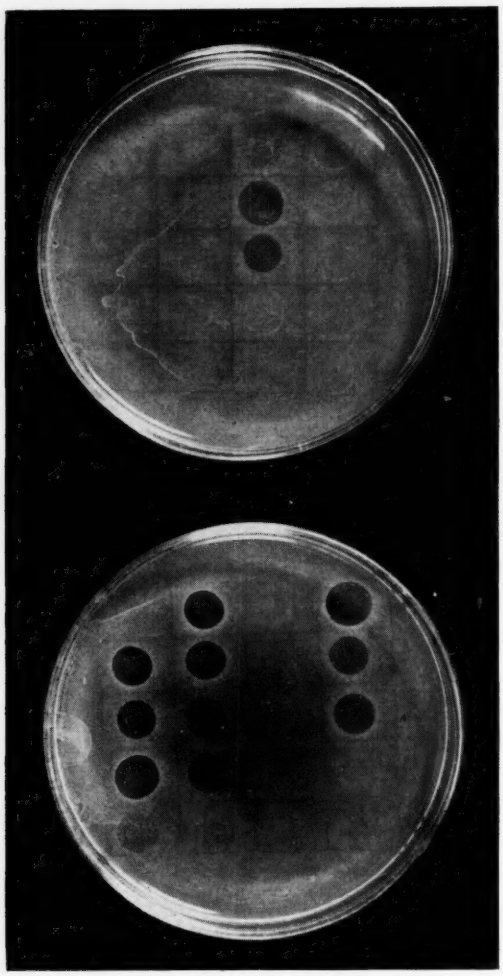

Fig. 3.—Bacteriophage patterns of two strains of *M. pyogenes* which illustrate varying degrees of lysis. On the left-hand plate, lysis from phage *3A* is 2+, and that with *3C* and *51* is 4+. On the right-hand plate lysis is 4+ except with phage *42B* (3+), *971* (2+), and a few individual plaques (1+) with phages *W*, *977*, and *1791*. Only lytic reactions of 2+ or greater were considered significant.

taken to avoid the tip of the needle contacting the agar surface of the plate. When all the phages had been spotted and had dried sufficiently, the plates were inverted and incubated at 30° C. overnight. Lysis was determined with the aid of an indirect bright light and a dark background. The results were recorded according to a 0 to 4+ scale, as follows: 4+ = complete confluent lysis, 3+ = confluent lysis with islands of growth, 2+ = more than fifty individual plaques of lysis, 1+ = less than fifty individual plaques, and 0 when no lysis was observed. These different degrees of lysis may be observed in Fig. 3. A strong reaction was considered 2+ or greater, and only strong reactions have been considered in the data presented.

RESULTS

Two thousand eighty-five strains of *M. pyogenes* isolated from 795 persons were tested with twenty staphylococcic bacteriophages. The anatomic source and the phage reaction of the strains are given in Table II. Five hundred forty-one strains showed strong lysis with one or more of the phages. This represents only 26 per cent of those tested. As indicated, one-quarter of the strains were coagulase-negative. If the coagulase-positive strains alone are considered, 35.2 per cent of the strains were lysed by one or more phages. It is apparent that the relatively low percentage of reacting strains is because of the large number which were obtained from the respiratory tract, particularly from the nose. Among the strains isolated from the stool, or from suppurative lesions, the proportion of strains lysed by one or more phages ranged from 52 to 67 per cent.

TABLE II. SUSCEPTIBILITY OF STAPHYLOCOCCI FROM 795 PERSONS TO TWENTY BACTERIOPHAGES

SOURCE	NUMBER OF STRAINS TESTED	NUMBER OF STRAINS COAGULASE-POSITIVE	PHAGE REACTION		PER CENT OF STRAINS PHAGE TYPED	
			STRONG LYSIS	WEAK OR NO LYSIS	TOTAL	COAGULASE-POSITIVE
Nose	1,276	834	241	1,035	19	29
Throat	557	459	178	379	32	39
Respiratory tract unclassified*	76	76	30	46	40	40
Stool	70	69	40	30	57	58
Osteomyelitis, wound	70	70	36	34	52	52
Abscess or skin	6	6	4	2	67	67
Blood and bone marrow	12	6	4	8	33	67
Others†	3	3	2	1	67	67
Unknown	15	13	6	9	40	46
Total	2,085	1,536	541	1,544	26	35.2

*Principally sputum.
†One strain each from urine, cerebrospinal fluid, and discharge from the eye.

The relationship of phage susceptibility to coagulase and alphahemolysin production is presented in Table III. While approximately one-third of the coagulase-positive strains showed strong lysis when exposed to phages, only a single coagulase-negative strain showed strong lysis. An attempt to correlate phage reaction with virulence, however (as measured by the production of alpha-hemolysin) indicated only a trend in the direction of more strains being typable when the alpha-hemolysin test was positive, and no clear separation between a positive and negative result.

TABLE III. THE ASSOCIATION OF PHAGE REACTION OF STAPHYLOCOCCI AND IN VITRO TESTS FOR VIRULENCE

	TOTAL NUMBER OF STRAINS	PHAGE REACTION	
		STRONG LYSIS	WEAK OR NO LYSIS
Coagulase test			
Positive	1,536	540	996
Negative	549	1	548
Alpha-hemolysin			
Positive	90	51	39
Doubtful	34	19	15
Negative	39	10	29

Among the 541 typable strains, 129 different reaction patterns were observed (Table IV). Two-thirds of the strains showed reaction patterns within the Phage Groups I, II, III, and Miscellaneous. In addition, four mixed groups were recognized. There were I-III, III-M, I-III-M, and I-II-III±M. Among the mixed groups, only the two former groups were represented by a significant number of strains. Nearly one-half of the typable strains gave patterns which were within Broad Phage Group III. While fifty-one individual patterns were observed among 259 strains in this group, 42.5 per cent of the strains were represented by the patterns *7/53* and *53*. The Mixed Phage Group I-III was the next most common, and included 17.2 per cent of the strains in twelve patterns. Slightly more than one-half of these strains were of a single pattern, *52/42B/47C*. Fourteen and two-tenths per cent of the strains reacted within Phage Group I. The predominant pattern was *52*. Mixed Group III-M differentiated 12.8 per cent of the strains and included forty individual patterns. The remaining phage groups each reacted with less than 5 per cent of the typable strains.

TABLE IV. DISTRIBUTION AND PATTERNS OF BACTERIOPHAGE SUSCEPTIBLE STRAINS OF *Staphylococci*

PHAGE GROUP	TYPABLE STRAINS		NUMBER OF PATTERNS	PREDOMINANT TYPE	
	NUMBER	PER CENT		PATTERN	PER CENT OF GROUP
Group I					
29/31/52	77	14.2	5	52	87.0
				29	7.8
Group II					
3A/3C/51	23	4.2	6	3C	56.5
				51	13.0
Group III					
6/7/42B/47/47B	259	47.9	51	7/53	21.6
47C/53/54				53	20.9
				47/54	5.6
				47/53	4.1
				54	4.1
Miscellaneous					
47A/W/977/1272	11	2.0	6	971	27.3
1791/971				W/977/1272/1791	27.3
				1272	18.2
Mixed Groups					
I-III	93	17.2	12	52/42B/47C	52.7
				52/47C	16.1
				52/42B	11.8
III-M	69	12.8	40	7/47/53/54/971	26.0
				54/971	5.8
				47/53/54/971	5.8
I-III-M	7	1.3	7		
I-II-III±M	2	0.4	2	29/3A/42B/53	
				29/51/54/971	
Total	541	100.0	129		

Table V indicates the frequency of cross reactions among phages from all groups for individual strains of *M. pyogenes*. One hundred ninety-seven strains were strongly lysed by a single bacteriophage, while 157 were strongly lysed by two phages, 101 by three, and the remaining 86 strains by four or more phages. Strains which were lysed by the phages in Groups I and II, except

31, were lysed only by these phages as frequently or more often than by any pattern of phages. Group II phages especially were limited in the breadth of their interactions. In contrast, the strains which were lysed by the phages of Groups III and M commonly were susceptible to other phages.

TABLE V. IDENTITY AND SPECIFICITY OF STAPHYLOCOCCIC BACTERIOPHAGES AMONG 541 UNKNOWN STRAINS OF *M. Pyogenes*

PHAGE		NUMBER OF STRAINS LYSED BY THE GIVEN PHAGE	TOTAL NUMBER OF LYSOGENIC PHAGES PER STRAIN										
GROUP	NUMBER		1	2	3	4	5	6	7	8	9	13	14
I	29	19	6	4	1	5	1	0	0	1	0	1	0
	31	7	0	2	1	2	1	0	0	0	0	1	0
	52	164	67	31	58	0	1	1	0	4	0	1	1
II	3A	6	2	2	1	1	0	0	0	0	0	0	0
	3C	18	13	4	1	0	0	0	0	0	0	0	0
	51	7	3	2	1	1	0	0	0	0	0	0	0
III	6	42	1	12	10	6	7	2	0	2	1	0	1
	7	146	11	65	22	12	23	4	2	4	1	1	1
	42B	100	6	18	55	3	3	3	4	5	1	1	1
	47	131	8	32	25	22	25	7	6	3	1	1	1
	47B	34	2	8	7	2	3	2	5	3	1	0	1
	47C	103	2	17	61	2	4	5	5	4	1	1	1
	53	234	59	82	27	21	27	7	4	4	1	1	1
	54	127	11	24	22	21	30	6	6	4	1	1	1
M	17	0	0	0	0	0	0	0	0	0	0	0	0
	W	19	1	2	1	4	4	2	2	1	0	1	1
	977	10	0	0	0	3	3	1	1	1	0	0	1
	1,272	19	2	1	2	4	4	2	1	1	0	1	1
	1,791	13	0	1	1	4	1	2	1	1	0	1	1
	971	70	3	7	7	11	28	4	5	2	1	1	1
Total number of strains			197	157	101	31	33	8	6	5	1	1	1

The patterns of association of lysogenic reactions of Group I phages with the other specific phages are demonstrated in Fig. 4. It is apparent that *29* and *31* react principally with one another. Indeed, in these studies *31* was never lysogenic for a strain which was not also lysed by *29*. Both phages were lysogenic occasionally, however, with all the other phages except those in Group II and *47A* and *47B*. Phage *52* was not lysogenic in association with the other Group I phages, but was essentially confined to association with *42B* and *47C*.

The lysogenic associations of Group II phages are shown in Fig. 5. With few exceptions, the association is restricted to other members of the group.

The lysogenic patterns for each of the Group III phages is shown graphically in Fig. 6. Certain characteristic patterns are immediately evident. These are *6/47/53*, *7/53*, *52/42B/47C*, *47/53/54*, and *7/47/53/54*. There are many minor variations, however, produced by association with other phages within Groups III, I, and M.

Among the miscellaneous group of phages, Fig. 7, there is frequent association one with another except in the case of phages *971* and *47A*. The latter failed to be strongly lysogenic for a single unknown strain of staphylococcus. Patterns of the Group M phages were primarily with Group III, but also quite frequently associated with Group I phages.

The variation in bacteriophage pattern introduced by storing strains of *M. pyogenes*, either by refrigeration or serial transfer in broth medium, and

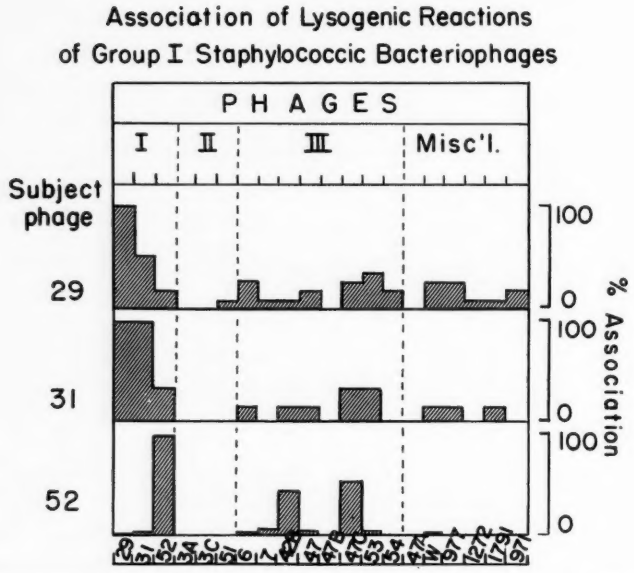

Fig. 4.—Association of lysogenic reactions of Group I staphylococcic bacteriophages.

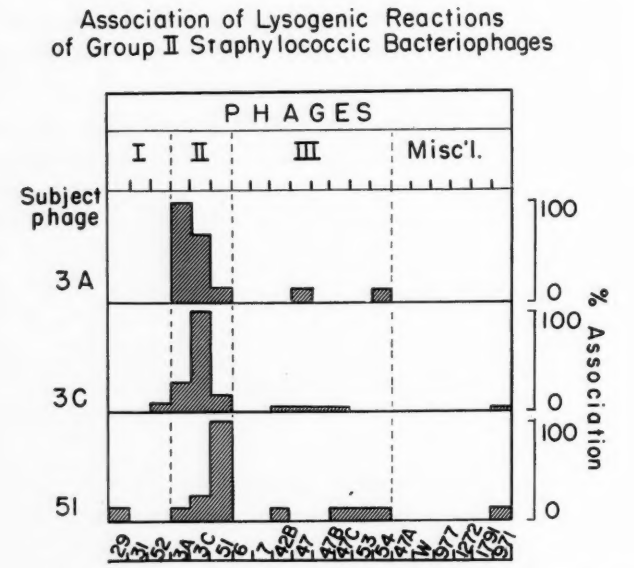

Fig. 5.—Association of lysogenic reactions of Group II staphylococcic bacteriophages.

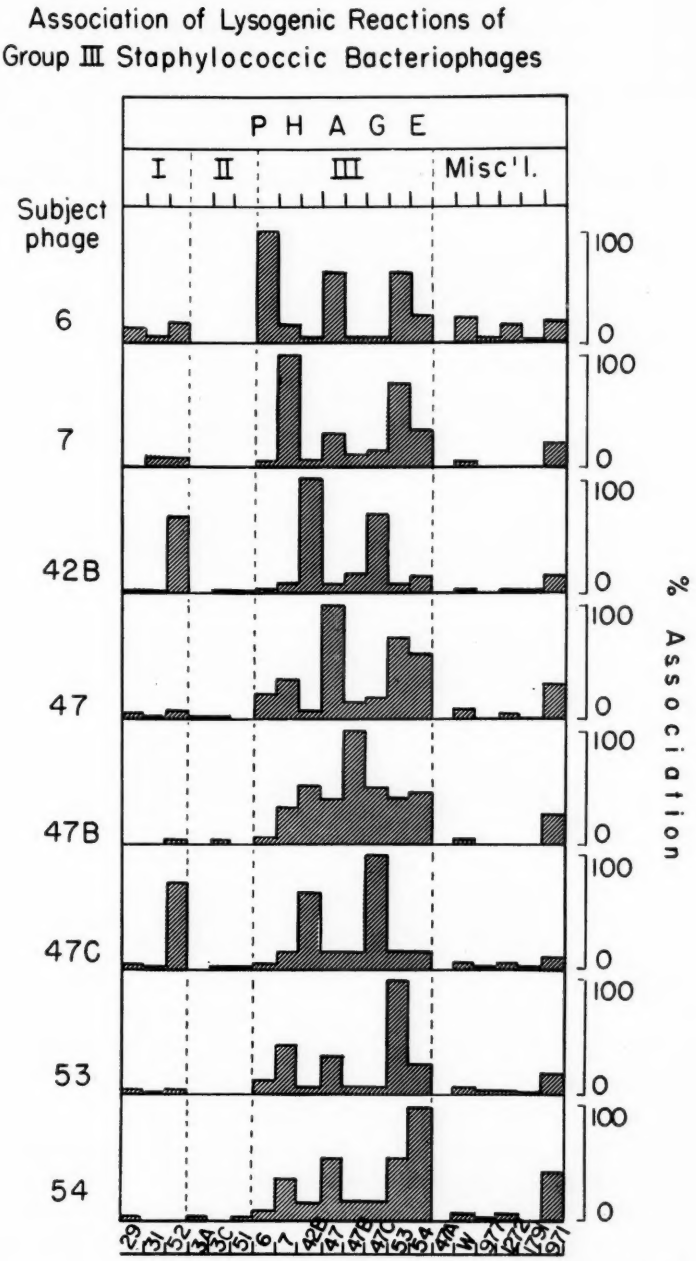

Fig. 6.—Association of lysogenic reactions of Group III staphylococcic bacteriophages.

in addition, the influence of different technicians and different lots of phage harvest, is presented in the left-hand column of Table VI. The variance among different colonies and different subcultures of the same strain is given in the center column of the table, and the influence of a concentrated inoculum of phage is compared with the results obtained using the critical test dose in the right-hand partition of the table.

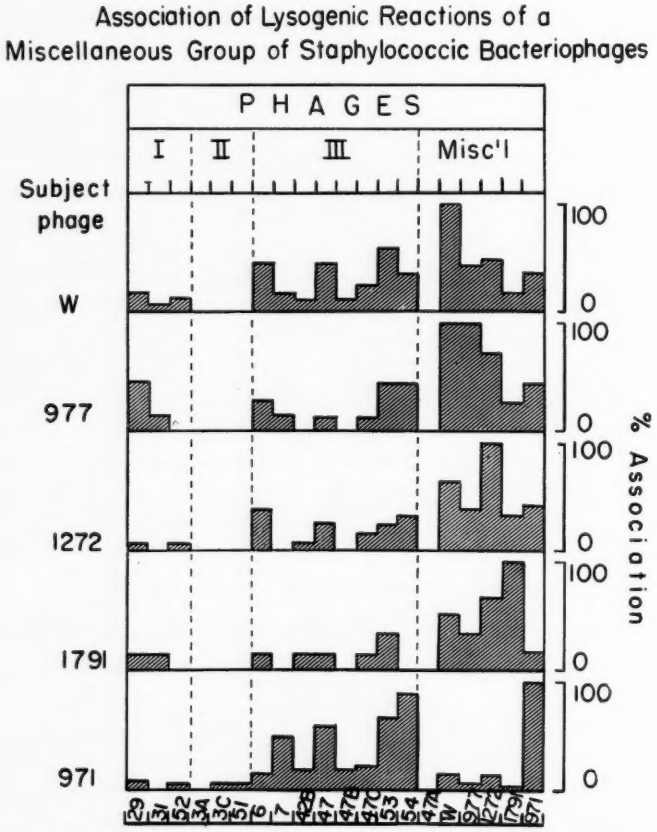

Fig. 7.—Association of lysogenic reactions of a miscellaneous group of staphylococcic bacteriophages.

Only 40 per cent of the strains tested with the R.T.D. of phage at different times had identical lysogenic patterns, but two-thirds differed by one phage or less in the same broad phage group. Approximately 25 per cent of the strains differed by more than a single phage reaction within one phage group. Lysis by a phage in a different group was observed upon retesting 3.7 per cent of strains, and 3.8 per cent of strains had become untypable with the R.T.D. of phage. Lytic patterns among different individual colonies from a single strain were identical more than two-thirds of the time, and 98.3 per cent reacted within the same broad phage group. The use of a concentrated suspension of phage compared with the C.T.D. in the testing of unknown strains increased the number of typable strains 51 per cent, and produced lysis in

TABLE VI. REPRODUCIBILITY OF THE BACTERIOPHAGE PATTERNS OF STRAINS MAINTAINED IN ARTIFICIAL MEDIUM, THE VARIABILITY AMONG DIFFERENT COLONIES, AND DIFFERENCES IN THE SUSCEPTIBILITY OF STRAINS TO DIFFERENT CONCENTRATIONS OF PHAGE

PHAGE REACTION	REPEAT TEST SAME CULTURE DIFFERENT TIMES* NO.	PER CENT OF SUBTOTAL	REPEAT TEST SAME CULTURE DIFFERENT COLONIES† NO.	PER CENT OF SUBTOTAL	COMPARISON SAME CULTURE C. T. D. VS. UNDILUTED PHAGE‡ NO.	PER CENT OF SUBTOTAL
Lysogenic Strains						
Identical	43	40.6	79	68.1	16	16.0
Identical ± 1 phage same group	28	26.4	26	22.4	11	11.0
Varied ± > 1 phage same group	27	25.5	9	7.8	19	19.0
Differ ± 1 phage other group	1	0.9	1	0.9	0	0
Differ ± > 1 phage other group	2	1.9	0	0	3	3.0
Both lysogenic	1	0.9	1	0.8	0	0
No phage in common One strain neg. at C.T.D.‡	4	3.8	0	0	51	51.0
Subtotal	106	100.0	116	100.0	100	100.0
Non-Lysogenic Strains						
Identical	27	—	15	—	47	—
Total	133		131		147	

*Most of the repetitions shown were performed by different technicians using different lots of phage. The cultures had been stored a few weeks to two years.

†Includes fifteen individual colonies from each of five strains, three individual colonies from each of nine strains, and the remainder represent different subcultures from a large inoculum.

‡Critical Test Dose is the highest dilution of phage which will produce complete confluent lysis of the propagating strain. Undiluted phage represents a 1:10 suspension of the phage harvest.

another broad phage group in only 3 per cent. Nearly one-half of the strains which did not type with the C.T.D., however, were also untypable with concentrated phage.

DISCUSSION

The techniques for recognition and propagation of staphylococcic bacteriophages have been advanced by many investigators and are now easily applied to the bacteriophage typing of staphylococci. The number of strains lysed by the phages used in this study was somewhat disappointing and is lower than the frequency obtained in some other series. As was indicated, strains isolated from the respiratory tract, and especially from the nose, seemed relatively nonsusceptible. Many of the persons from whom these strains were isolated were household contacts of patients admitted to the hospital and, therefore, represented normal persons. Williams and Rippon[14] found a total of 58 per cent of 252 strains from the noses of normal persons in England were lysed by one or more phages, and Rountree[15] found 70 per cent of 387 strains isolated from the noses of normal persons in Australia to be phage susceptible. In this country, Blair and Carr[16] were able to type 53 per cent of 539 strains from various sources. These data include only coagulase-positive strains. From our material presented, it may be assumed that coagulase-negative strains are resistant to

the phages used. The one typed strain which was reported as coagulase-negative was not available for verification and may have been found to be coagulase-positive as were two or three other apparently negative strains which were lysed by phages. That phage susceptibility is a biologic characteristic of the strain not dependent upon virulence per se is suggested by the lack of correlation with the test for alpha-hemolysin.

The number of strains successfully identified and differentiated by phage reactions is obviously a function of the specific bacteriophages and the concentration in which they are used. It has been suggested that phages derived from the same locale as the strains being tested may be superior with regard to the number of strains one is able to type. Although sufficiently widespread trials of this hypothesis have not been performed to endorse the view, it should not be ignored as a hypothesis. The phages propagated by the workers in England, however, have been found to produce fairly similar frequency and similar patterns of lysis wherever they have been used. Consequently the Subcommittee on Bacteriophage Typing of Staphylococci at the 1953 International Congress of Microbiologists tentatively agreed on a "basic set" of nineteen staphylococcic bacteriophages which they recommended for routine testing. In the present study, several members of the "basic set" were not available. It is possible that inclusion of the other members might have significantly increased the number of strains which were lysed. The R.T.D. rather than a concentrated inoculum of phage was employed throughout the study for uniformity, since preliminary observations indicated no significant differences, and the opinion was held that the use of undiluted phage would complicate interpretation by the production of side reactions. In view of the accumulated experience, more satisfactory results might have been achieved with regard to the number of typable strains observed if a concentrated phage inoculum had been used.

A number of other modifications of the technique for routine testing of unknown strains were of minor importance. A medium free of glucose was used to diminish the overgrowth of phage-resistant cells which occasionally produced difficulty in evaluating the degree of original lysis. A slightly alkaline agar was preferred because a sharper contrast between the plaques and surrounding growth appeared. A broth transfer of the unknown culture gave similar results when the incubation period was varied between six and eighteen hours at 37° C. The latter time was used in routine typing for convenience. A soft agar medium was found to be preferable, but little effect was observed from varying the agar content between 0.9 and 2.5 per cent. Addition of urea in hypertonic concentrations, as suggested by Bronfenbrenner[17] for *Escherichia coli* bacteriophages, was not helpful, nor was elimination of sodium chloride from the medium. Continual incubation of the culture at 30° C., after it had been spotted with phage, produced more satisfactory results than the technique of brief incubation at 37° C. followed by overnight standing at room temperature.

The distribution of the 541 typed strains from our studies among the broad phage groups may be compared with the series previously reported from England,[14] Australia,[15] and New York.[16] The 14.2 per cent in Group I is lower

than 22 per cent, approximately 18 per cent, and 38 per cent, respectively, in the other reports. Similarly, 4.2 per cent in Group II is low compared to 12 per cent, approximately 26 per cent, and 24 per cent, respectively, in the previous studies. The infrequency of strains among Groups I and II is compensated by a greater per cent of strains in Group III. We observed 47.9 per cent as compared to percentages of 33, 23, and 26 in the other series. In addition, a greater number of our strains were classified among the mixed groups. This may be explained in part by the use of specific phages among the miscellaneous group that differed from those employed by others, and in part by the increased occurrence of some strains which may have gained predominance within a particular area of one of the hospitals from which our strains were obtained.

It is apparent from the association patterns of lysogenic reactions shown in Figs. 4 to 7, that classification into the broad phage groups is not satisfactory for identification of specific strains. If, on the other hand, each pattern of lysis were to be designated as a specific type, 129 types of staphylococci would have been reported from this study alone. It has reasonably been recommended, therefore, that when individual bacterial strains are reported or referred to, the pattern of lysis be recorded by listing the specific phages which produce strong lysis, e.g., *6/47/53/54*, rather than by listing a type. The technique of bacteriophage typing of staphylococci is not as specific or reproducible as a serologic reaction such as is available for the grouping and typing of streptococci, and because of the multiplicity of cross reactions among the phages, typing of staphylococci is less reliable than phage typing of salmonella species. Satisfactory confidence limits were obtained, however, for similarity among different colonies of a single culture. Less confidence can be placed upon the differences observed for cultures tested at different times, or after storage. Nevertheless, if one applies the variance of a strong reaction with plus or minus one specific phage from among a broad phage group, which is a rather strict criterion for similar identity, reproducible results should be obtained for well over two-thirds of the typable strains of *M. pyogenes*. Within the same broad phage group, reproducibility can be attained in 92.5 per cent of retests.

SUMMARY

a. The results of the bacteriophage typing of 2,085 strains of staphylococci are given, along with the technique that has been used in typing.

b. Five hundred forty-one strains (26 per cent of all strains tested or 35.2 per cent of coagulase-positive strains) were lysed by one or more of the twenty bacteriophages used. Only one coagulase-negative strain showed a significant degree of lysis. The alpha-hemolysin test correlated fairly well with lysis by bacteriophages.

c. The typed strains were distributed among the phage groups as follows: Group I, 14.2 per cent; Group II, 4.2 per cent; Group III, 47.9 per cent; Group I-III, 17.2 per cent; Group III-M, 12.8 per cent; and remaining 3.7 per cent among three additional groups.

d. The interactions of the phage types have been discussed, and also the reproducibility of the phage tests under varying conditions. Upon retesting, reaction in a different phage group was observed in 3.7 per cent of strains, and 3.8 per cent of strains had become untypable with the routine test dose of bacteriophage.

The invaluable technical assistance in these studies contributed by Winifred Harmon, Joseph Seto, Ethel Rosenthal, Greta Long, Jeanette Norsen, and Gayle Hager, is gratefully acknowledged.

REFERENCES

1. Burnet, F. M., and Lush, D.: The Staphylococcal Bacteriophages, J. Path. & Bact. **40**: 455-469, 1935.
2. Williams, S., and Timmins, C.: An Investigation of the Source of Staphylococcal Infections in Acute Osteomyelitis, M. J. Australia **2**: 687, 1938.
3. Fisk, R. T.: Studies on Staphylococci. I. Occurrence of Bacteriophage Carriers Amongst Strains of *Staphylococcus aureus*, J. Infect. Dis. **71**: 153, 1942.
4. Fisk, R. T.: Studies on Staphylococci. II. Identification of *Staphylococcus aureus* by Means of Bacteriophage, J. Infect. Dis. **71**: 161, 1942.
5. Fisk, R. T., and Mordvin, Olga E.: Studies on Staphylococci. III. Further Observations on Bacteriophage Typing of *Staphylococcus aureus*, Am. J. Hyg. **40**: 232, 1944.
6. Wilson, G. S., and Atkinson, J. D.: Typing of Staphylococci by the Bacteriophage Method, Lancet **1**: 647, 1945.
7. Dowling, H. F., Lepper, M. H., and Jackson, G. G.: Observations on the Epidemiological Spread of Antibiotic-Resistant Staphylococci, With Measurements of the Changes in Sensitivity to Penicillin and Aureomycin, Am. J. Pub. Health **43**: 860, 1953.
8. Fisk, Alan: The Technique of the Coagulase Test for Staphylococci, Brit. J. Exper. Path. **21**: 311, 1940.
9. MacFarlan, A. M.: Incidence of Pathogenic Staphylococci in the Nose, Brit. M. J. **2**: 939, 1938.
10. Williams, R. E. O., and Cowan, S. T. Personal communication.
11. Williams, R. E. O., and Rippon, J. E.: Bacteriophage Typing of *Staphylococcus aureus*, J. Hyg. **50**: 320-353, 1952.
12. Colbeck, J. C.: An Extensive Outbreak of Staphylococcal Infections in Maternity Units, Canad. M. A. J. **61**: 557-668, 1949.
13. Swanstrom, M., and Adams, M. H.: Agar Layer Method for Production of High Titer Phage Stocks, Proc. Soc. Exper. Biol. & Med. **78**: 372-375, 1951.
14. Williams, R. E. O., and Rippon, J. E.: Bacteriophage Typing of Strains of Staphylococcus Aureus From Various Sources, Lancet **1**: 510-513, 1953.
15. Rountree, P. M.: Bacteriophage Typing of Strains of Staphylococci Isolated in Australia, Lancet **1**: 514-516, 1953.
16. Blair, J. E., and Carr, M.: The Bacteriophage Typing of Staphylococci, J. Infect. Dis. **93**: 1-13, 1953.
17. Bronfenbrenner, J.: Mechanism of Lysis of Bacteria by Bacteriophage (Abst.), Science **116**: 517, 1952.

BACTERIOPHAGE TYPING OF STAPHYLOCOCCI

II. Epidemiologic Studies Among Patients, Household Contacts, and Hospital Personnel

George Gee Jackson, M.D., Harry F. Dowling, M.D., and Mark H. Lepper, M.D.
Chicago, Ill.

BACTERIOPHAGE typing of staphylococci offers a reasonably good method for identifying individual strains of *Micrococcus pyogenes* and a very satisfactory method of classifying strains within broad phage groups based upon the patterns of lysis produced by the various phages.[1] Through the use of the bacteriophage technique, it has been possible to examine the homogeneity of staphylococci as they are derived from different population groups, different sources of isolation, and different time periods. Comparable data have not been available for the other tests which are commonly employed for the identification and classification of *M. pyogenes*. Since coagulase-negative strains of *M. pyogenes* are not susceptible to lysis in any significant number by the bacteriophages that were employed, the data obtained from the present study concern only potentially pathogenic strains.

Observations upon the transfer of specific strains of *M. pyogenes* from one person to another often can be documented by the use of bacteriophage typing with a high probability of validity. We have used this approach to make certain observations on the epidemiologic spread of staphylococci among patients, household contacts, and hospital personnel. Some aspects of the investigations have been presented previously.[2, 3] The present report will attempt to relate the epidemiologic findings to the bacteriophage-groups of staphylococci involved.

MATERIALS AND METHODS

Sterile swabs were employed to obtain specimens from the noses and throats. These were either streaked immediately upon agar plates containing 6 per cent human blood, or placed in a tube with a small amount of nutrient broth and transported to the laboratory. There was never more than two hours' time between the taking of cultures and the seeding of plates. Patients were selected for culture in rotation according to the sequence of their admission to the Chicago Municipal Contagious Disease Hospital. Specimens were obtained for culture in the admitting room prior to hospital admission, regardless of the type of illness present, and two or three times each week after entry to the hospital wards. Culture specimens from patients in other hospitals have been included in some aspects of the analysis. In all, 215 patients yielded phage-typable staphylococci from one or more culture specimens. Approximately one-half of the patients were from a single hospital, referred to previously.

From the Department of Medicine, Research and Educational Hospitals, and Chicago Municipal Contagious Disease Hospital, University of Illinois College of Medicine, Chicago, Ill.
Received for publication, April 1, 1954.

TABLE I. DISTRIBUTION OF PHAGE SUSCEPTIBLE STRAINS OF *M. Pyogenes* ACCORDING TO THE SOURCE OF ISOLATION

PHAGE GROUP*	EXPECTED PERCENT†	SOURCE												TOTAL NUMBER STRAINS
		NOSE		THROAT		UPPER RESP. UNCLASSIFIED‡		STOOL		OSTEOMYELITIS		OTHER		
		NO.	%	NO.	%	NO.	%	NO.	%	NO.	%	NO.	%	
I	14.2	40	16.6	28	15.7	7	23.4	2	5.0	–	–	–	–	77
II	4.2	15	6.2	4	2.2	3	10.0	1	2.5	–	–	–	–	23
III	47.9	111	46.1	70	39.4	9	30.0	17	42.5	36	100.0	16	100	259
M	2.0	4	1.7	5	2.8	–	–	2	5.0	–	–	–	–	11
Mixed Groups														
I-III	17.2	42	17.4	48	27.0	3	10.0	–	–	–	–	–	–	93
III-M	12.8	25	10.4	20	11.2	6	20.0	18	45.0	–	–	–	–	69
I-III-M	1.3	3	1.2	2	1.1	2	6.6	–	–	–	–	–	–	7
I-II-III±M	0.4	1	0.4	1	0.6	–	–	–	–	–	–	–	–	2
Total	100	241	100	178	100	30	100	40	100	36	100	16	100	541

*The phage groups were composed of the following specific phages: I = *29/31/52*; II = *3A/3C/51*; III = *6/7/42B/47B/47C/53/54*; M = W/*977/1272/1791/971/47A*

†Assuming no predilection of any phage group for any source.

‡Principally sputum.

When patients retained or acquired a persistently positive culture of *M. pyogenes* in the hospital, each of the household contacts of the patient was studied, in so far as possible, by means of nose and throat swabs prior to discharge of the patient to his home. All of the members who permitted cultures to be taken from the nose and throat were subsequently studied at 2, 4, 6, and occasionally 8, 12, and 16 weeks after the time of the patient's discharge. Strains of *M. pyogenes* which were lysed by bacteriophage were isolated from one or more household contacts in twenty-six family units. The majority of these studies were made between September, 1951, and March, 1952.

Nose and throat cultures were obtained from members of the hospital personnel who entered the wards on which the patients were housed during their hospital stay early in the period of the investigation, again near its conclusion, and subsequently at intervals of approximately one month. Phage-typable strains of *M. pyogenes* were obtained on one or more occasions from fifty-one members among the hospital staff.

The techniques employed in identification and bacteriophage typing of strains of *M. pyogenes* have been described in detail in another publication.[1]

RESULTS

Altogether, 541 strains of *M. pyogenes* reacted with one or more of the twenty bacteriophages employed to give a pattern of strong lysis. Among these, 423 strains were derived from the environmental study that centered around the patients in one hospital. In the analysis of the results, bacteriophage patterns of individual strains have not been given in most instances because of the multiplicity of minor variations in patterns, instead the phage patterns have been considered as belonging to one of eight broad phage groups.

In Table I is shown the distribution of the strains of *M. pyogenes* isolated from among the 795 persons from whom cultures were obtained, according to the different sources of isolation and the phage reactions of the strains. Column 1 indicates the proportion of the strains which would be expected in each phage group if the distribution were completely random. Comparison of the observed distribution with that expected by means of Chi-square analysis indicated that the over-all distribution of strains from the different sources was not significantly different from a random distribution except in the mixed Groups I-III and III-M. If, however, all the strains obtained from the respiratory tract are grouped together, in contrast to all the strains that were derived from other sources, there is statistically a very significant increase above the expected frequency of occurrence of strains from Phage Group I in the respiratory tract. Strains of Phage Group II occurred more frequently than expected in the nose and less frequently in the throat and other sites. The number of observations was too few, however, to have statistical significance. A comparison of the strains among Group III that were found in the respiratory tract with those found elsewhere, indicates a significantly lower proportion from the respiratory tract than would be expected from random distribution ($p = 0.05$). Among the mixed groups, strains in Phage Group I to III had a significant predilection for the throat, and strains among Phage Group III-M a very significant predilection for the gastrointestinal tract.

Five different patterns of lysis were observed among the strains of Group I isolated from the respiratory tract, although 90 per cent of the strains be-

longed to type *52*. Six lytic patterns were found among the strains of Group II, and 59 per cent of these were type *3C*. Among 189 respiratory tract strains in Group III, 49 patterns of lysis were observed, and among 70 strains from other sources, 15 patterns were seen. All except two patterns were common to Group III strains from either source. Lysis by phages *53* or *7/53* was characteristic for 42.5 per cent of the Group III strains from all sources, but strains lysed by these phages alone comprised 58 per cent of the Group III strains that occurred in the throat. Thus, in contrast to Phage Group III as a whole, these two types were found in the throat as frequently as in the nose. On the other hand, strains lysed by phages *53* or *7/53* alone comprised only 35 per cent of Group III strains from the stool. Phages *54*, *6/47/54*, and *6/47/53/54* characterized most of the other strains isolated from the stool. In the mixed Group I to III there were twelve patterns of lysis. With the exception of a single phage pattern, the distribution between the nose and throat was similar. Two patients were pharyngeal carriers of strain *52/7/47C*, which was not found in the nose of any person. Forty lytic patterns occurred among strains of the mixed Group III-M, but only two major variations were due to the specific M phages. These were the presence or absence of lysis by phages *971* or *W*. Among the Group III-M strains isolated from the stool, the former was lysogenic for all eighteen strains, and the *W* phage was lysogenic for only one.

TABLE II. DISTRIBUTION OF PHAGE-SUSCEPTIBLE STRAINS OF STAPHYLOCOCCI AMONG PATIENTS, HOUSEHOLD CONTRACTS, AND HOSPITAL PERSONNEL

PHAGE GROUP	EXPECTED PER CENT*	PATIENTS		HOUSEHOLD CONTACTS		HOSPITAL PERSONNEL		TOTAL
		NO. STRAINS	%	NO. STRAINS	%	NO. STRAINS	%	NO. STRAINS
I	16.8	47	16.4	17	25.8	7	9.8	71
II	4.7	10	3.5	8	12.0	2	2.8	20
III	45.9	133	46.5	22	33.4	39	55.0	194
M	2.1	7	2.4	1	1.5	1	1.4	9
Mixed Groups								
I-III	21.1	59	20.6	16	24.2	14	19.7	89
III-M	8.3	27	9.5	0	0	8	11.3	35
I-III-M	0.9	2	0.7	2	3.1	0	0	4
I-II-III	0.2	1	0.4	0	0	0	0	1
Total	100	286	100	66	100	71	100	423

*Assuming equal distribution among all three years.

In Table II, strains of *M. pyogenes* derived from patients in a single hospital, their household contacts, and the personnel of the hospital, are tabulated according to the identification of the strains within broad phage groups. The mean per cent of strains expected in each phage group, if random distribution had prevailed, is shown in the first column. Some trends toward lack of uniform distribution are readily apparent. Strains among Phage Groups I and II are more prevalent among household contacts than among either hospital patients or personnel, whereas strains of Group III are more prevalent among personnel and patients. From the number of strains observed, however, the abnormal distribution is not statistically significant by Chi-square analysis.

The most consistent finding of all was that the distribution of the strains recovered from patients lies midway between the frequency observed among household contacts and hospital personnel. Therefore, the initial cultures obtained from patients in the admitting room of the hospital were compared with

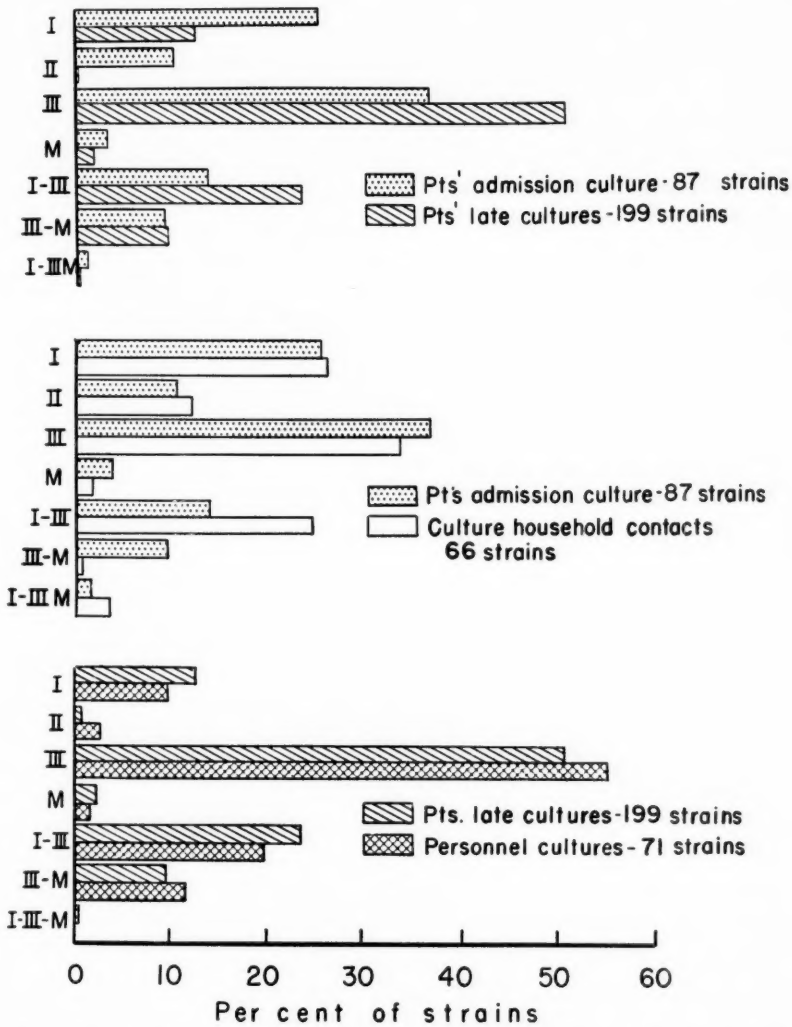

Fig. 1.—Comparison of the strains of *M. pyogenes* obtained from patients, their household contacts, and hospital personnel, according to the broad phage groups to which they belong.

those obtained after admission to the hospital ward. The lack of similarity between the strains isolated at these different times is shown graphically in the top panel of Fig. 1. In contrast, there is great similarity between the strains of *M. pyogenes* cultured on admission and those cultured from household contacts, as shown in the center panel of Fig. 1. Likewise, the similarity is striking between the strains of *M. pyogenes* isolated from patients after entry to the hospital wards, and those found among hospital personnel as

illustrated in the bottom panel of Fig. 1. In view of these findings, the distribution of strains of *M. pyogenes* between hospital personnel and household contacts was analyzed by adding the strains recovered from patients upon admission to those of the household contacts, and strains recovered subsequent to admission were added to those from hospital personnel. This reveals that the more frequent occurrence of strains from Phage Groups I and II among household contacts is statistically significant, $p = 0.05$ and 0.02, respectively, as compared with the lower incidence among hospital personnel. On the other hand, strains among Broad Phage Group III and Mixed Groups I to III and III-M are not significantly different in their distribution between household contacts and personnel.

TABLE III. DISTRIBUTION OF PHAGE-SUSCEPTIBLE STRAINS OF STAPHYLOCOCCI IN A SINGLE HOSPITAL ACCORDING TO THE YEAR OF ISOLATION

PHAGE GROUP	EXPECTED PER CENT*	1951		1952		1953		TOTAL NUMBER STRAINS
		NO.	%	NO.	%	NO.	%	
I	16.8	19	16.5	34	22.4	18	11.5	71
II	4.7	4	3.5	12	7.9	4	2.5	20
III	45.9	39	33.9	50	32.9	105	67.4	194
M	2.1	2	1.7	5	3.3	2	1.3	9
I-III	21.1	45	39.2	37	24.4	7	4.5	89
III-M	8.3	5	4.3	11	7.2	19	12.2	35
I-III-M	0.9	1	0.9	3	1.9	0	0	4
I-II-III±M	0.2	0	0	0	0	1	0.6	1
Total	100	115	100	152	100	156	100	423

*Assuming uniform distribution of all groups among the population.

In Table III, strains of *M. pyogenes* have been classified according to phage groups and the year in which they were isolated. Two trends have statistical significance. These are the increased occurrence of strains in Group III in the year 1953, and the diminishing frequency of strains in the Mixed Group I-III with the passage of time. Examination of the specific phage patterns revealed no change in the identity of strains in Groups I or III. In Group II, strains lysed by type *51* were predominant in the year 1952, but almost disappeared in 1953 when strains lysed by type *3A* appeared. A progressive decrease in strains of a single pattern, *52/42B/47C*, is responsible for the decrease observed in the Mixed Group I-III.

The exchange of strains of *M. pyogenes* with the environment for persons among the three categories studied was observed by comparison of the phage patterns of strains isolated from successive cultures from the same anatomic site. The results are shown for patients, household contacts, and hospital personnel, respectively, in Tables IV, V, and VI. Among the patients, 55 per cent of strains isolated from the nose were identical in successive cultures. An additional 16 per cent were probably of the same origin, while 6.5 per cent were strains of different origin. In the throat, 85.6 per cent were probably the same, and 6.2 per cent were different. These results were midway between the exchange observed for household contacts and for hospital personnel. Among the former group, 70 per cent of the strains of *M. pyogenes* from the

TABLE IV. SUCCESSIVE CULTURES FROM INDIVIDUAL PATIENTS:

PHAGE REACTION*	SAME SITE DIFFERENT TIMES			DIFFERENT SITE	DIFFERENT SITE
	NOSE	THROAT	OTHER	SAME TIME	DIFFERENT TIME
Identical phage pattern	35	67	24	47	68
Identical ± one phage in the same group†	9	16	10	6	40
Varies more than ± one phage in the same group†	12	7	8	6	69
Differs ± one phage in another group	2	1	0	0	3
Differs more than one phage in another group	0	5	0	3	0
No phage in common	4	1	0	3	7
Total	62	97	42	65	187

*Lines 1 and 2 indicate organisms probably from the same strain.
Line 3 indicates organisms of indeterminate origin, possibly the same strain.
Line 4 indicates organisms of indeterminate origin, probably different strains.
Lines 5 and 6 indicate organisms from different strains.
†Including the miscellaneous phage *971* with Broad Group III.

TABLE V. SUCCESSIVE CULTURES FROM INDIVIDUAL HOUSEHOLD CONTACTS

PHAGE REACTION*	SAME SITE DIFFERENT TIMES			DIFFERENT SITE	DIFFERENT SITE	DIFFERENT CONTACT
	NOSE	THROAT	OTHER	SAME TIME	DIFFERENT TIME	SAME TIME
Identical phage pattern	24	12	0	10	5	5
Identical ± one phage in the same group†	2	0	0	0	1	5
Varies more than ± one phage in the same group†	4	0	0	0	0	2
Differs ± one phage in another group	3	0	0	0	0	0
Differs more than one phage in another group	1	0	0	0	1	0
No phage in common	0	0	0	0	0	1
Total	34	12	0	10	7	13

*Lines 1 and 2 indicate organisms probably from the same strain.
Line 3 indicates organisms of intermediate origin, possibly the same strain.
Line 4 indicates organisms of indeterminate origin, probably different strains.
Lines 5 and 6 indicate organisms from different strains.
†Including miscellaneous phage *971* in Broad Group III.

nose, and all of the strains from the throat were identical on successive cultures. Among hospital personnel, only 38.4 per cent of strains from the nose on successive culture produced identical phage patterns and an equal number showed strains of different origin. Only among patients were strains of a different phage pattern cultured from different anatomic sites at any one time. As indicated in Fig. 2, this was sometimes accounted for by the fact that the patient had a family strain in the nose and had acquired a different strain in the throat. Hospital personnel differed from the other groups by the infre-

quency with which the same strain was recovered from different sites in successive cultures. As shown in Table V, strains that occurred simultaneously among different members of a household were remarkably similar in their phage patterns.

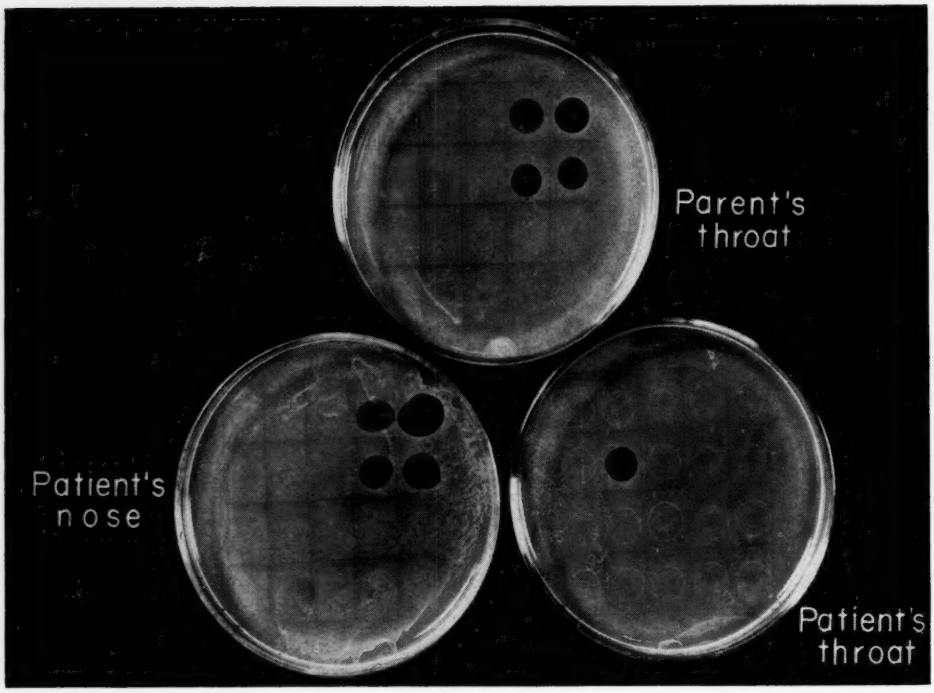

Fig. 2.—The identification by bacteriophage typing of different strains of *M. pyogenes* in the nose and throat of a patient, and the presence of one of these strains in the throat of his parent, cultured simultaneously.

TABLE VI. SUCCESSIVE CULTURES FROM INDIVIDUALS AMONG THE HOSPITAL PERSONNEL

PHAGE REACTION*	SAME SITE DIFFERENT TIMES		DIFFERENT SITE	DIFFERENT SITE
	NOSE	THROAT	SAME TIME	DIFFERENT TIME
Identical phage pattern	10	0	1	1
Identical ± one phage in the same group†	3	1	3	1
Varies more than ± one phage in the same group†	2	0	0	0
Differs ± one phage in another group	1	0	0	0
Differs more than one phage in another group	1	0	0	1
No phage in common	9	1	0	7
Total	26	2	4	10

*Lines 1 and 2 indicate organisms probably from the same strain.
Line 3 indicates organisms of indeterminate origin, possibly the same strain.
Line 4 indicates organisms of indeterminate origin, probably different strains.
Lines 5 and 6 indicate organisms from different strains.
†Including miscellaneous phage *971* in Broad Group III.

DISCUSSION

In the presentation of results, the strains of *M. pyogenes* have been classified according to four broad phages groups which were made up on the basis of frequent association of lytic reactions among the component phages, and four mixed phage groups constructed upon the frequency of cross reactions among the phage-typable strains of *M. pyogenes*.[1] This does not indicate that all of the strains in any one group are identical. Numerous patterns of phage lysis were observed within each broad phage group. The grouping of strains according to their reactions in broad phage groups, however, facilitates analysis for epidemiologic interpretation, and also aids in comparison with similar data reported from England and Australia.

The lack of uniform representation of the phage groups among coagulase-positive staphylococci isolated from different anatomic sites and different types of pathologic lesions is gaining importance as a result of the documentation of similar observations in England, Australia, and the United States.[4-6] The expansion of such data will bring the study of staphylococci a little nearer to the advantages already obtained by the ability to type pneumococci and streptococci.

In England, Williams and associates[4] found that strains belonging to Group I were most common among staphylococci cultured from nasal swabs from normal healthy persons and, furthermore, comprised 56 per cent of strains from persons with an acute fulminating staphylococcal pneumonia. Rountree[5] studied inpatients and outpatients in a metropolitan area in Australia and found a large proportion of strains from the respiratory tract belonged to Group I, but a larger proportion belonged to Group III. This significant discrepancy from the results obtained in England was assigned to a difference in the populations studied, an assumption that is supported by our data. Our data also are obtained primarily from metropolitan areas, largely from a single hospital, and in contrast to the other studies, a single person is usually represented by more than one culture. Nevertheless, the preponderance of strains belonging to Phage Group III is comparable to that observed by Rountree.[5] The results of Williams and associates[4] are corroborated in that Group I strains occurred significantly more frequently in the respiratory tract than would be expected from random distribution, and strains from the Mixed Group I-III were found exclusively in the respiratory tract, particularly in the throat.

Strains of *M. pyogenes* susceptible to the phages of broad Group II were found infrequently in the present study. This observation is entirely in keeping with what might have been expected, since the previous studies in England[4] and Australia,[5] as well as the results of Blair and Carr[6] in this country, indicated that strains in Group II were found most commonly from boils, otitis, abscesses, and osteomyelitis. These sources were inadequately represented in our data.

Food poisoning in Great Britain appears almost entirely to be caused by strains of *M. pyogenes* in Phage Group III. In our data, the strains isolated from stools were from patients with staphylococcic enteritis as a complication of antibiotic treatment. Approximately one-third of the typed strains were isolated by each of three different groups of workers, in three medical centers, the Boston City Hospital,* the Mayo Clinic,† and our own hospitals. Strains of Group III and Mixed Phage Group III-M were most common. Ninety-three per cent belonged to Group III, III-M, or M. In contrast, strains of Group III-M were not recovered from a household contact in a single instance. The strains from each hospital formed eight or nine specific phage patterns and only two or three were common to any two of the hospitals studied. The patterns observed were very similar to those shown to be associated with well-authenticated outbreaks of food poisoning in England,[4] while strains with other patterns in Group III showed a diminished tendency to occur in the stools of patients with dysentery, and might have appeared coincidentally with their presence in the upper respiratory passages, as noted by Williams and associates.[4]

The preponderance of Group III strains shown to be associated with osteomyelitis in our studies is somewhat contrary to the results of others and might be accounted for by the fact that all the patients had chronic osteomyelitis, and the strains obtained from the wounds in such cases are more likely a reflection of hospital strains than the ones causing the primary lesion.

The irregular distribution in the frequency with which strains of *M. pyogenes* of different phage groups were found among household contacts and hospital personnel is of importance. The results have great similarity in the low incidence of Group II strains to those obtained by Rountree for several hospitals in Australia.[5] The distribution is strongly influenced by the sensitivity of the strains to penicillin, and this aspect will be discussed separately, along with additional data.[7] The distribution of strains among the years also has probably been influenced by the introduction of new antibiotic agents, and the population sampled.

The role played by hospital personnel in acquiring and disseminating strains of *M. pyogenes* among hospital patients, and in turn the exchange of strains among patients and household contacts, has been elaborated in previous reports by means other than bacteriophage typing of the strains.[2, 3, 8] The previous findings are reinforced by the current observations. Different strains of *M. pyogenes* were acquired in the noses and throats of hospital personnel between successive cultures more than five times as often as among household contacts. This quantitative estimate may be erroneously high, however, because the intervals between cultures often were greater among personnel than among household contacts. From a small number of cases, it was previously observed that approximately 10 per cent of patients transferred hospital-acquired strains to another family contact after they had

*Obtained from Dr. M. Finland, Throndike Memorial Laboratory.
†Obtained from Dr. W. H. Heilman, Division of Bacteriology, Mayo Clinic.

been discharged from the hospital.[2] In support of this, the occurrence of Group III strains recovered from among household contacts increased after the patient was discharged from the hospital, although the number of observations is too few to have statistical significance. Since the hospital in which these studies were performed functions as an isolation unit, precautionary measures to prevent the spread of infection were practiced by the staff, and the patients were adequately spaced or placed in separate cubicles. One may expect, therefore, to find the personnel in a general hospital to be contributing to an even greater extent in the exchange of strains among patients and consequently in the community.

SUMMARY AND CONCLUSION

The sources from which 541 strains of staphylococci were derived have been studied in relation to the broad phage group in which the strains belonged. Group I strains tended to be obtained more frequently from the respiratory tract, and Group III strains less frequently than would be expected in a purely random distribution. Group I-III strains showed a significant predilection for the throat, and Group III-M strains a significant predilection for the intestinal tract.

Individual patterns of lysis were studied in relation to the source of strains. Ninety per cent of the Group I strains obtained from the respiratory tract were lysed by phage *52*. Among strains in Group III, types *53* and *7/53* were commonly obtained from the respiratory tract, while type *6/47/53/54* was most frequent in the stool.

In an epidemiologic study of the patients and personnel from a single hospital and the household contacts of the patients, it was found that strains from Groups 1 and II were found more frequently among household contacts, and from Group III among hospital personnel, while strains from patients tended to fall in both groups.

The proportion of strains belonging to Group III that were recovered during the year 1953 increased in frequency as compared with the two preceding years, whereas the frequency of strains in Group I-III diminished progressively from 1951 to 1953.

All of the strains obtained from the throats, and 70 per cent of the strains from the noses, of the household contacts were grouped in the same groups on successive cultures. Among hospital personnel, 38.4 per cent of strains from the nose were identical on successive cultures by the same criterion. Among patients, 85.6 per cent of strains cultured from throats and 55 per cent of the strains cultured from noses were of the same group on successive cultures.

Of the strains isolated from the stools of patients with staphylococcic enteritis, which followed antibiotic therapy, 93 per cent belonged to Group III, III-M, M.

The invaluable technical assistance in these studies contributed by Winifred Harmon, Joseph Seto, Ethel Rosenthal, Greta Long, Jeanette Norsen, and Gayle Hager is gratefully acknowledged.

REFERENCES

1. Jackson, G. G., Dowling, H. F., and Lepper, M. H.: Bacteriophage Typing of Staphylococci. I. Technique and Patterns of Lysis. J. LAB. & CLIN. MED. **44**: 14-28, 1954.
2. Dowling, H. F., Lepper, M. H., and Jackson, G. G.: Observations on the Epidemiological Spread of Antibiotic-Resistant Staphylococci, With Measurements of the Changes in Sensitivity to Penicillin and Aureomycin, Am. J. Pub. Health **43**: 860, 1953.
3. Lepper, M. H., Dowling, H. F., Jackson, G. G., and Hirsch, M. M.: Epidemiology of Penicillin- and Aureomycin-Resistant Staphylococci in a Hospital Population, Arch. Int. Med. **92**: 40-50, 1953.
4. Williams, R. E. O., Rippon, J. E., and Dowsett, L. M.: Bacteriophage Typing of Strains of *Staphylococcus aureus* from Various Sources, Lancet **1**: 510-513, 1953.
5. Rountree, P. M.: Bacteriophage Typing of Strains of Staphylococci Isolated in Australia, Lancet **1**: 514-516, 1953.
6. Blair, J. E., and Carr, M.: The Bacteriophage Typing of Staphylococci, J. Infect. Dis. **93**: 1-13, 1953.
7. Jackson, G. G., Lepper M. H., and Dowling H. F.: Bacteriophage Typing of Staphylococci. III. Relationship to Antibiotic Sensitivity and Resistance. J. LAB. & CLIN. MED. **44**: 41-50, 1954.
8. Lepper, M. H., Moulton, B., Dowling, H. F., Jackson, G. G., and Kofman, S.: Epidemiology of Erythromycin-Resistant Staphylococci in a Hospital Population. Effect on Therapeutic Activity of Erythromycin, Antibiotics Annual 1953-54 (Proceedings of the Symposium on Antibiotics), 1953, Medical Encyclopedia, Inc., p. 308.

BACTERIOPHAGE TYPING OF STAPHYLOCOCCI

III. Relationship to Antibiotic Sensitivity and Resistance

George Gee Jackson, M.D., Mark H. Lepper, M.D., and
Harry F. Dowling, M.D.
Chicago, Ill.

THE patterns of lysis among strains of staphylococci produced by a set of twenty different bacteriophages have permitted us to classify coagulase-positive strains into four broad phage groups, four mixed phage groups, and nontypable strains.[1] Although strains with many specific phage patterns are present in each phage group, the classification into broad groups appears to have epidemiologic significance related to the incidence of strains among persons in different catagories of the population, the proportion of strains from each phage group isolated from different anatomic sites, and the pathologic lesions commonly encountered with staphylococci in different phage groups.[2] In the present paper, the classification of strains into broad phage groups will be employed to examine the relationship of phage reaction and the inhibition of growth produced in vitro by penicillin, chlortetracycline, and erythromycin.

MATERIALS AND METHODS

The sources of clinical material and the techniques of isolating and phage typing strains of staphylococci have been presented in detail previously.[1, 2]

Antibiotic sensitivity tests were performed in 100 by 13 mm. test tubes. Serial twofold dilutions of each antibiotic were made in 0.5 ml. of tryptose phosphate broth (pH 7.0), and an inoculum of 1.5 ml. of a 1:100 dilution of an 18- to 24-hour broth culture (10,000 to 1,000,000 organisms per milliliter in the final dilution) of the test strain was added. The tube with the highest dilution of the antibiotic showing complete absence of gross turbidity after 18 to 24 hours of incubation at 37° C. was recorded as the minimal inhibitory concentration (M.I.C.), and this value was used as the antibiotic sensitivity of the strain.

The antibiotics, potassium penicillin G, chlortetracycline hydrochloride,* and erythromycin hydrochloride† were prepared in a concentrated aqueous solution and aliquots were dispensed into individual test tubes. These stock solutions were kept in a frozen state at −20° C. The contents of one tube of each antibiotic were thawed just prior to use. The potency of each agent and the accuracy of the dilutions were controlled for each set of determinations by performing a microbiologic assay with a standard strain of *Bacillus circulans* for penicillin, *Bacillus cereus* for chlortetracycline, and *Streptococcus C-203* for erythromycin.

RESULTS

The minimal inhibitory concentration of penicillin was determined for 427 strains of phage-typable coagulase-positive staphylococci; and also for 907 additional coagulase-positive strains, and 505 coagulase-negative strains that were not lysed by the bacteriophages employed. Almost all of these strains were similarly tested with chlortetracycline and approximately one-half of them were tested with erythromycin. The results are given in Table I.

From the Department of Medicine, Research and Educational Hospitals, and Chicago Municipal Contagious Disease Hospital, University of Illinois College of Medicine, Chicago, Ill.

Received for publication, April 1, 1954.

*Chlortetracycline was supplied by Lederle Laboratories Division of American Cyanamid Co., Pearl River, N. Y.

†Erythromycin was furnished by Abbott Laboratories, North Chicago, Ill.

TABLE I. THE RELATION OF THE EFFECT OF ANTIBIOTICS UPON THE GROWTH OF STAPHYLOCOCCI TO THEIR REACTIVITY TO COAGULASE AND TO BACTERIOPHAGES

ANTIBIOTIC	INHIBITING CONCENTRATION	(A) NOT PHAGE TYPABLE; COAGULASE-NEGATIVE		(B) NOT PHAGE TYPABLE; COAGULASE-POSITIVE		(C) PHAGE TYPABLE; COAGULASE-POSITIVE		SIGNIFICANCE P		
		NO.	%	NO.	%	NO.	%	A—B	A—C	B—C
Penicillin	1 unit or less	258	51.1	374	41.3	100	23.4	$<.05$	$<.001$	$<.001$
	10 units	65	12.9	80	8.8	44	10.3	-	-	-
	100 units	79	15.6	159	17.5	88	20.6	-	-	-
	250 units	53	10.5	162	17.8	81	19.0	$<.01$	$<.05$	-
Total	>250 units	50	9.9	132	14.6	114	26.7	-	$<.001$	$<.001$
		505		907		427				
Chlortetracycline	1 μg or less	130	25.8	330	36.5	124	29.1	$<.05$	-	-
	10 μg	186	37.0	292	32.3	89	20.8	-	.001	$<.05$
	100 μg	129	25.6	129	14.3	66	15.5	$<.001$	$<.05$	-
	250 μg	50	9.9	130	14.4	107	25.2	-	$<.001$	$<.01$
Total	>250 μg	9	1.7	23	2.5	40	9.4	-	$<.01$	$<.001$
		504		904		426				
Erythromycin	1 μg or less	176	81.1	292	68.7	204	75.0	$<.05$	-	-
	10 μg	21	9.7	32	7.5	14	5.1	-	-	-
	100 μg	8	3.7	15	3.5	1	0.4	-	-	$<.05$
	250 μg	5	2.3	5	1.2	4	1.5	-	-	-
Total	>250 μg	7	3.2	81	19.1	49	18.0	$<.001$	$<.001$	-
		217		425		272				

The percentage of strains completely inhibited by concentrations of penicillin less than 1 unit per milliliter was significantly greater among coagulase-negative strains than among those that were coagulase-positive. Among the latter, there were significantly more strains sensitive to penicillin in the group that was not phage-typable. Conversely, a concentration of 250 units of penicillin per milliliter failed to inhibit a significantly greater number of phage-typable strains than other coagulase-positive or coagulase-negative strains.

Chlortetracycline most effectively inhibited coagulase-positive staphylococci that were not phage-typable. A significantly smaller percentage of coagulase-negative strains was inhibited by less than 1 unit per milliliter of chlortetracycline. As was the case with penicillin, significantly more of the phage-typable coagulase-positive strains required 250 μg or more of chlortetracycline per milliliter to inhibit growth than was observed among strains that were either coagulase-negative or coagulase-positive and nontypable.

One microgram of erythromycin per milliliter inhibited the majority of strains of staphylococci in all three groups. In this concentration, however, significantly more coagulase-negative strains were inhibited than nonphage-typable coagulase-positive staphylococci. More significant, however, was the larger percentage of coagulase-positive strains, regardless of phage reaction, that were resistant to inhibition by 250 μg per milliliter than was observed among the coagulase-negative strains. Phage-typable strains of intermediate sensitivity to erythromycin were significantly less frequent than was observed among other coagulase-positive strains, but such strains were relatively rare in both groups.

The distribution of the phage-typable strains according to the inhibiting concentrations of penicillin and broad phage groups is presented in Fig. 1. Groups represented by less than twenty strains are not shown. It is immediately apparent that there was not a uniform distribution of strains belonging to different phage groups with regard to their inhibition by penicillin. The significance of these differences will be considered later. The striking features observed were the high proportion of sensitive strains in Group II, and the high proportion of resistant strains in the Phage Groups III, III-M, and I-III. Among the latter groups, there was a uniform distribution of strains inhibited by intermediate concentrations of penicillin. On the other hand, the strains in Phage Group I were divided more evenly between those inhibited by low concentrations of penicillin and those resistant to high concentrations. This is similar to, and intermediate between, the distribution of nontypable coagulase-positive strains and coagulase-negative strains.

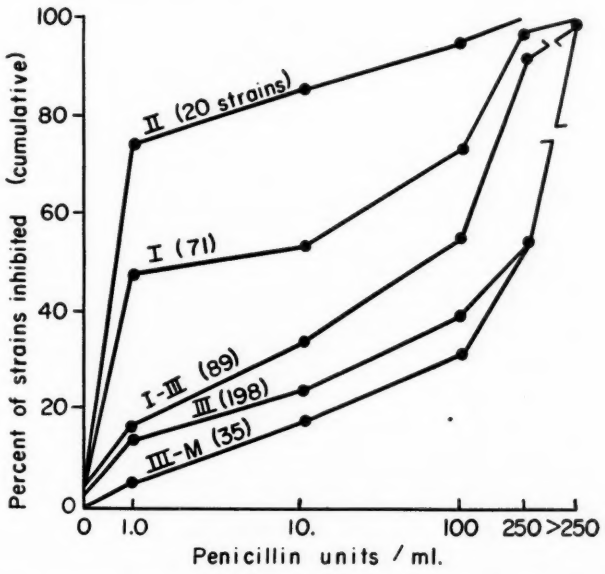

Fig. 1.—The penicillin sensitivity of strains of *M. pyogenes* belonging to different phage groups.

The distribution of strains within any one phage group cannot be accounted for by the sensitivity or resistance to penicillin of strains with any particular phage pattern. The majority of strains in Group I, however, were lysed by phage *52* alone, and nearly all of the strains in the Mixed Group I-III were lysed by some combination of the phages *52*, *42B*, and *47C*.

The minimal inhibitory concentration of chlortetracycline for the strains in different phage groups is given in Fig. 2. As was the case for penicillin, the distribution among the phage groups was not uniform, and the highest proportion of strains inhibited by low concentrations of chlortetracycline was found in Group II. A large proportion of the strains belonging to the Mixed Phage Group I-III were not inhibited by high concentrations of chlortetracycline.

Strains in the Phage Groups I, III, and III-M showed a rather symmetrical distribution with regard to inhibition by increasing concentrations of chlortetracycline.

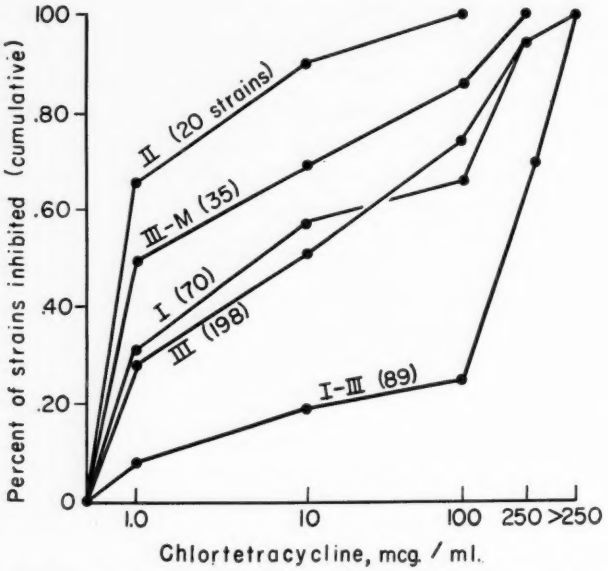

Fig. 2.—The chlortetracycline sensitivity of strains of *M. pyogenes* belonging to different phage groups.

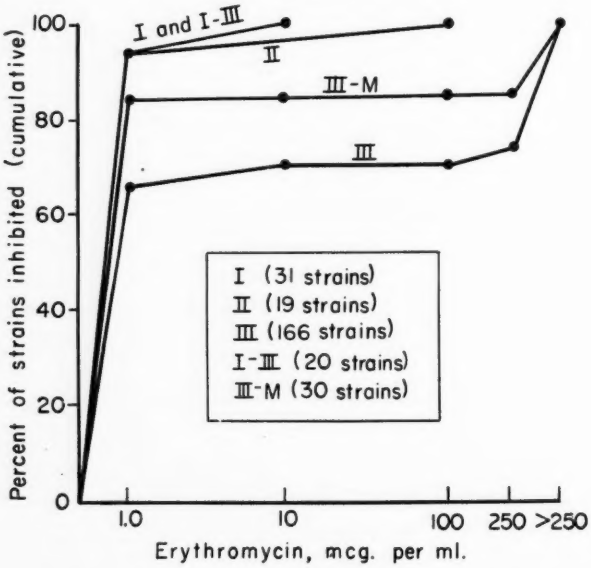

Fig. 3.—The erythromycin sensitivity of strains of *M. pyogenes* belonging to different phage groups.

The cumulative distribution curves for strains among the different phage groups, according to the minimal inhibitory concentrations of erythromycin, are shown in Fig. 3. Nearly all of the strains in Phage Group I and Mixed Group

I-III were inhibited by low concentrations of erythromycin. Strains not inhibited by erythromycin were found only in Phage Group III and Mixed Group III-M. The horizontal line in the zone of intermediate concentrations of erythromycin indicates the absence of strains inhibited in this area, and suggests that inhibition by erythromycin tends to be an all-or-none phenomenon for strains in these phage groups.

TABLE II. THE RELATIONSHIP OF PHAGE GROUPS AND SENSITIVITY OR RESISTANCE TO ANTIBIOTICS

AGENT	TOTAL STRAINS TESTED	PHAGE GROUP	ANTIBIOTIC-SENSITIVE STRAINS* NUMBER‡ EXPECTED	ANTIBIOTIC-SENSITIVE STRAINS* NUMBER OBSERVED	ANTIBIOTIC-SENSITIVE STRAINS* P	ANTIBIOTIC-RESISTANT STRAINS† NUMBER‡ EXPECTED	ANTIBIOTIC-RESISTANT STRAINS† NUMBER OBSERVED	ANTIBIOTIC-RESISTANT STRAINS† P
Penicillin	427	I	16.6	34	<.05	32.4	19	-
		II	4.7	15	<.05	9.1	1	<.05
		III	46.5	28	<.05	90.5	120	<.05
		M	2.1	4	-	4.1	2	-
		I-III	20.8	14	-	40.6	27	-
		III-M	8.2	2	<.05	16.0	24	-
		Other	1.1	3	-	2.3	2	-
			100.0	100		195.0	195	
Chlortetracycline	426	I	20.4	23	-	24.1	24	-
		II	5.8	12	-	6.9	0	<.05
		III	58.0	57	-	68.7	51	<.05
		M	2.6	5	-	3.1	0	-
		I-III	25.6	7	<.01	30.5	66	<.05
		III-M	10.2	17	-	12.0	5	-
		Other	1.4	2	-	1.7	1	-
			124.0	124		147.0	147	
Erythromycin	272	I	25.6	29	-	5.1	0	<.05
		II	15.4	17	-	3.1	0	-
		III	118.0	109	-	34.9	48	<.05
		M	3.0	3	-	0.6	0	-
		I-III	16.7	19	-	3.3	0	-
		III-M	23.6	25	-	5.7	5	-
		Other	1.7	2	-	0.3	0	-
			204.0	204		53.0	53	

*Growth inhibited by 1 unit or 1 μg per milliliter or less.
†Required 250 or more units or μg per milliliter to inhibit growth.
‡Based on the proportion of strains tested from each phage group.

A measure of the statistical significance of the lack of uniformity in the distribution of staphylococci in different phage groups, with regard to inhibition by antibiotics, is given in Table II. The number of strains inhibited by one unit or microgram of one of the agents, the antibiotic-sensitive strains, and the number of strains which required 250 or more units or micrograms to produce inhibition, the antibiotic-resistant strains, were observed and compared with the number of strains expected in each category based on the number tested in each phage group. Significance was determined by Chi-square analysis. There was a significantly greater frequency of penicillin-sensitive strains among Phage Groups I and II, and a significantly lower incidence of sensitive strains in Phage Group III and Mixed Group III-M. Conversely, significantly fewer strains among Phage Group II were penicillin-resistant, and the number of strains resistant to penicillin among Phage Group III was significantly greater than expected.

The significant findings with regard to chlortetracycline were the small number of sensitive strains and the increased incidence of resistant strains in the Mixed Phage Group I-III. In contrast to penicillin, the occurrence of resistant strains in Group III is significantly lower than expected by random distribution, and the trend in Group III-M is toward increased susceptibility to chlortetracycline. The absence of resistant strains among Group II is statistically significant.

All of the strains resistant to erythromycin belonged to either Phage Group III or III-M, the number among Group III was increased to a statistically significant level, while the absence of such strains in Phage Group I also had statistical significance.

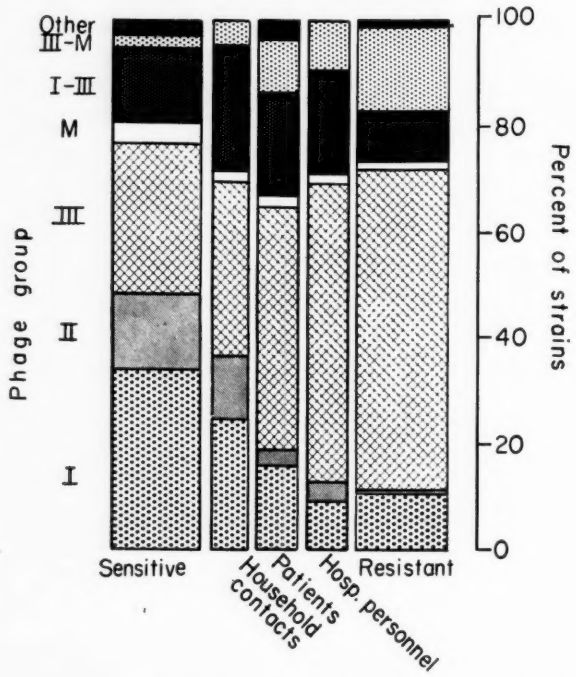

Fig. 4.—The representation of phage groups among 100 strains of *M. pyogenes* inhibited by 1 unit or less of penicillin per milliliter, the sensitive strains, and among 195 strains requiring 250 units or more of penicillin per milliliter to inhibit growth, the resistant strains, compared with the representation of phage groups among strains of *M. pyogenes* isolated from patients, household contacts, and hospital personnel.

The representation of each of the phage groups among 100 strains of staphylococci that were effectively inhibited by one unit of penicillin is illustrated by the left-hand bar in Fig. 4, and, compared with the occurrence of the same phage groups among patients, their household contacts, and hospital personnel. In the right-hand bar, the representation of the phage groups among 195 strains of staphylococci that required 250 units or more for inhibition is shown. It may be seen that the composition by phage reaction of the penicillin-sensitive strains most nearly simulates that observed among household contacts, while the phage composition among penicillin-resistant strains is more compar-

able to that observed among hospital personnel. The phage groups of Staphylococci obtained from hospital patients was intermediate between these two groups of persons. Similar comparisons for chlortetracycline and erythromycin indicate the same trends less clearly.

DISCUSSION

The increasing clinical importance of antibiotic-resistant staphylococci is documented by numerous reports in the literature.[3] On the other hand, some reports have called attention to the fact that antibiotic-resistant strains are increased primarily in hospital areas rather than in the community at large, and that certain types of lesions are still commonly associated with penicillin-sensitive strains of *Micrococcus pyogenes*.[4] The technique of bacteriophage testing has made it possible to examine the similarity of different strains of staphylococci within the species in relationship to another biologic characteristic of the strain. Barber and Whitehead[5] demonstrated that strains of the Phage Group *6/47* (Group III) were most commonly isolated from hospital personnel and comprised a large proportion of the penicillin-resistant strains. This finding was borne out in the present study. A later investigation in the same hospital, reported by Elwood,[6] indicated that a large proportion of the strains in the Phage Group *29/31/52* (Group I) and *3* (Group II) were still sensitive to penicillin, while more than one-half of the strains belonging to the Phage Group *6/7/47* (Group III) were penicillin-resistant strains.

In a report upon the results of bacteriophage testing of staphylococci in Sweden, Laurell and Wallmark[7] found strains of some phage types practically all penicillin-resistant, and strains belonging to another phage type nearly all sensitive to penicillin. Since the bacteriophages used in testing were not identical to those we used or those used by the British workers, one cannot make direct comparisons; strains belonging to the phage type which corresponds to the *6/47* group of the British (Group III), however, were resistant to penicillin.

In Australia, Rountree[8] found 66 to 80 per cent of the penicillin-resistant strains of staphylococci from different sources belonged to Phage Group III, and the majority of strains in Phage Groups I and II were penicillin-sensitive. In Canada, Leyton[9] observed the appearance of strains of staphylococci that were resistant to chlortetracycline, and studied the relationship of phage susceptibility and antibiotic resistance. Over a period of thirty-two months, the percentage of strains sensitive to chlortetracycline decreased from ninety-nine to seventy-two. Only one strain, 8 per cent, that belonged to Phage Group *51* (Group II) was not inhibited by chlortetracycline; whereas eleven strains, 55 per cent, that belonged to Phage Group *6/47B* (Group III) were chlortetracycline-resistant. The author, however, did not feel that there was a straightforward relationship between sensitivity to antibiotics and phage type, and suggested that there are potentially resistant organisms in all phage groups. Lowbury and Topley,[10] however, observed some correlation between phage type and chlortetracycline sensitivity in the strains of staphylococci isolated from burns.

A recent report by Fusillo and associates[11] from the District of Columbia correlated phage distribution and antibiotic-resistant staphylococci. The results agree very closely with those presented here, although the authors did not state what limits were used in classifying strains as "sensitive" or "resistant," and the strains were accumulated only from patients in a hospital environment. Nearly all of the phage-typable strains resistant to antibiotics were found in Phage Group III, and 74.5 per cent of all penicillin-resistant strains belonged to this phage group. The increased number of strains in Group III that were resistant to streptomycin, however, was not statistically significant, nor was the increase in tetracycline-resistant strains. In Group II, antibiotic-resistant strains were rare, but some resistant strains were found in each broad phage group regardless of the antibiotic that was used.

From the similarity in the experience that has accumulated from several laboratories in distant geographic areas, and from the statistically significant differences in the number of antibiotic-sensitive and antibiotic-resistant strains among the phage groups in our own results, the indication is quite clear that the emergence of resistant strains in vivo is not the same among Staphylococci of different phage groups. It is generally accepted that the emergence of resistant strains is a consequence of selection of resistant mutants among the bacterial population.[12] One might suspect, therefore, that penicillinase-producing mutants have occurred with decreased frequency among organisms of certain phage groups, or, alternatively, that there has been less selection by antibiotics among strains in these phage groups because of their distribution in the population or the type of lesions which they produce. It is possible that the nuclear material of some strains of *M. pyogenes* in Group III have an increased facility for adaptation or genetic change with regard to a variety of stimuli or methods of selection. Studies regarding these hypotheses are presently being conducted.

The majority of reports in this country upon the inhibition of staphylococci by antibiotics have considered all coagulase-positive strains as a homogeneous group. Coagulase-negative staphylococci are generally agreed to behave independently in regard to inhibition by antibiotics. In the data reported, antibiotic-resistant strains for all three antibiotics were least frequent among coagulase-negative organisms, and, with the exception of chlortetracycline, the largest number of sensitive strains occurred in the coagulase-negative group. Considering coagulase-positive organisms, Finland and co-workers[3, 13] have clearly demonstrated the increase in the number of strains resistant to one or more antibiotics with the passage of time at a single large institution. The distribution of the phage-typable coagulase-positive strains from the data presented here corresponds very closely, with regard to penicillin sensitivity, to that reported by Finland and Haight[3] for strains isolated at approximately the same time. By bacteriophage typing, however, the composition of the staphylococci studied by us has been shown to include strains of different phage groups which differed from one another to a significant degree in their sensitivity to antibiotics.

Strains belonging to different phage groups did not necessarily behave in the same manner toward each of the antibiotics tested. In this respect, the strains of Mixed Phage Group III-M were found to be most resistant to penicillin and relatively more susceptible to chlortetracycline, and strains in Group I-III were most resistant to chlortetracycline but sensitive to erythromycin. The strains belonging to Broad Phage Group II, however, had a relatively large proportion of strains that were effectively inhibited by all three antibiotics, and strains among Broad Phage Group III were relatively resistant to all three antibiotics.

Strains of Mixed Phage Group I-III were common at the Chicago Municipal Contagious Disease Hospital, but disappeared almost completely following the introduction of erythromycin into the therapeutic regimens in the wards, and erythromycin-resistant strains have not yet appeared in that phage group. The distribution of strains according to their inhibition by erythromycin as shown, however, represents only the present status, and erythromycin-resistant strains are presently being encountered with increased frequency.[14]

It is noteworthy that the phage groups to which one-half of the penicillin-sensitive strains belong, i.e., Groups I and II, were found significantly more frequently among household contacts than among hospital personnel, while the Phage Groups III, I-III, and III-M, to which 90 per cent of the strains resistant to penicillin belong, show no statistically significant difference among the three population categories studied. This suggests that the high proportion of resistant strains observed among hospital personnel is primarily because of the elimination of sensitive organisms, and that, among the general population, potentially resistant strains occur to nearly the same extent.

SUMMARY

The relationship of antibiotic sensitivity and phage typing for 1,839 strains of *M. pyogenes* has been studied.

The minimal inhibitory concentration of penicillin has been determined for 505 coagulase-negative, and 1,334 coagulase-positive, strains, of which 427 were lysed by one or more bacteriophage. The sources of the strains were several hospitals in different parts of the country, but predominantly one hospital in Chicago. Strains from personnel, patients, and home contacts are included. Among the coagulase-negative organisms, 51.1 per cent were sensitive to 1 unit per milliliter or less of penicillin, among the nontypable coagulase-postitive strains, 41.3 per cent, and among the phage-typable strains, 23.4 per cent were similarly sensitive. On the other hand, the percentages which were not inhibited by 250 units per milliliter of penicillin were 9.9, 14.6, and 24.7 per cent, respectively, for the three groups.

The in vitro inhibition of growth of staphylococci by penicillin was found to be significantly different among the phage groups. Strains in Phage Groups I and II had a greater percentage of penicillin-sensitive strains, and Groups III and III-M a greater percentage of resistant strains than expected.

Similarly, the minimal inhibitory concentration of chlortetracycline has been determined for 504 coagulase-negative, and 1,330 of the coagulase-positive strains, including 426 of the phage-typable strains. There were more strains resistant to 250 μg per milliliter of chlortetracycline in the phage-typable group, 9.4 per cent, than in the coagulase-positive nontypable group, 2.5 per cent, and coagulase-negative group, 1.7 per cent. There were fewer resistant strains in Phage Groups II and III, but more than expected in Phage Group I-III.

The minimal inhibitory concentration of erythromycin was determined for 217 coagulase-negative, and 697 coagulase-positive strains, including 272 which were phage-typable. The incidence of strains inhibited by 1 μg per milliliter or less in the three groups was 81.1, 68.7, and 75.0 per cent, respectively. On the other hand, 3.2, 19.1, and 18.0 per cent, respectively, were resistant to 250 μg per milliliter of the drug. Phage Group I had significantly fewer erythromycin-resistant strains than expected, and Phage Group III, more than expected. All of the resistant strains were found to be in Phage Groups III and III-M.

The invaluable technical assistance in these studies contributed by Winifred Harmon, Joseph Seto, Ethel Rosenthal, Greta Long, Jeanette Norsen, and Gayle Hager is gratefully acknowledged.

REFERENCES

1. Jackson, G. G., Dowling, H. F., and Lepper, M. H.: Bacteriophage Typing of Staphylococci. I. Technique and Patterns of Lysis. J. Lab. & Clin Med. **44**: 14-28, 1954.
2. Jackson, G. G., Dowling, H. F., and Lepper, M. H.: Bacteriophage Typing of Staphylococci. II. Epidemiologic Studies Among Patients, Household Contacts and Hospital Personnel. J. Lab. & Clin. Med. **44**: 29-40, 1954.
3. Finland, M., and Haight, T. H.: Antibiotic Resistance of Pathogenic Staphylococci, Arch. Int. Med. **91**: 143-158, 1953.
4. Sherris, J. C., and Florey, M. E.: Relation of Penicillin Sensitivity in Staphylococci to Clinical Manifestations of Infection, Lancet **1**: 309, 1951.
5. Barber, M., and Whitehead, J. E. M.: Bacteriophage Types in Penicillin-Resistant Staphylococcal Infection, Brit. M. J. **2**: 565-569, 1949.
6. Elwood, J. S.: The Penicillin Sensitivity and Phage Types of Staphylococci Isolated From Hospital Patients, J. Hyg. **49**: 263-267, 1951.
7. Laurell, G., and Wallmark, G.: Studies on *Staphylococcus aureus pyogenes* in a Children's Hospital. III. Results of Phage-typing and Tests for Penicillin Resistance of 2,474 Strains Isolated From Patients and Staff, Acta Path. et Microbiol. scandinav. **32**: 438-447, 1953.
8. Rountree, P. M.: Bacteriophage Typing of Strains of Staphylococci Isolated in Australia, Lancet **1**: 514-516, 1953.
9. Leyton, G. B.: Some Observations on Staphylococci and Their Resistance to Antibiotics, Canad. M. A. J. **67**: 20-22, 1952.
10. Lowbury, E. J. L., and Topley, E.: Chemotherapy for *Staphylococcus aureus* in Burns, Lancet **1**: 1036, 1952.
11. Fusillo, M. H., Roerig, R. N., Metzger, J. F., and Ernst, K. F.: Phage Typing Antibiotic-Resistant Staphylococci, Am. J. Pub. Health **44**: 317, 1954.
12. Demerec, M.: Origin of Bacterial Resistance to Antibiotics, J. Bact. **56**: 63, 1948.
13. Finland, M., Frank, P. F., and Wilcox, C.: In Vitro Susceptibility of Pathogenic Staphylococci to Seven Antibiotics, Am. J. Clin. Path. **20**: 326-334, 1950.
14. Lepper, M. H., Moulton, B., Dowling, H. F., Jackson, G. G., and Kofman, S.: Epidemiology of Erythromycin-Resistant Staphylococci in a Hospital Population. Effect on Therapeutic Activity of Erythromycin. Antibiotics Annual 1953-54 (Proceedings of the Symposium on Antibiotics) New York, 1953, Medical Encyclopedia, Inc., p. 308.

A SIMPLE PROCEDURE FOR THE IDENTIFICATION OF THE GENUS SALMONELLA BY MEANS OF A SPECIFIC BACTERIOPHAGE

WILLIAM B. CHERRY, PH.D., BETTY R. DAVIS, B.A., PHILIP R. EDWARDS, PH.D., AND RALPH B. HOGAN, M.D.
ATLANTA, GA.

THE conventional procedures for the identification of Salmonella are based on both biochemical and serologic studies.[1] Usually, serum containing both somatic and flagellar antibody is employed for screening of these organisms. Such sera are available commercially but are quite expensive for surveys where large numbers of cultures must be tested.

Experiments in which a single selected bacteriophage is employed for generic identification of Salmonella are reported here. The method is rapid, simple, and quite specific. One worker can easily test 200 to 400 cultures in one working day including the four-hour period necessary for incubation of the broth prior to the application of phage. In terms of time required per test performed, phage identification is very rapid, although testing cannot be done directly on colonies appearing on isolation plates as is possible with polyvalent Salmonella serum. Based on the current price of dehydrated media and of commercial polyvalent agglutinating serum, the use of serum for the identification of these organisms is approximately ten times as costly as is the use of phage. The cost of the phage is negligible since a supply sufficient to last for years can be prepared within a few hours. The "technical" time, however, as with other biologic tests is the most expensive factor.

METHODS

A selected bacteriophage of high titer was applied in concentrated form to inoculated areas on plates of selective media. The plates were incubated at 37° C. for a period of six to eighteen hours and were then examined for evidence of lysis of the bacteria. Although many bacteriophages were isolated and tested, none proved as satisfactory as the phage described as "O-1" in the paper of Felix and Callow.[2] This phage was known to have lytic action on Salmonella belonging to many somatic groups, but the specificity of its action and its potentialities as a diagnostic tool have not been reported. The phage used in these experiments was propagated to a high titer in broth at 37° C. using *S. paratyphi B* (phage type 1) as the host organism. The broth was prepared by adding 20 Gm. of nutrient broth dehydrated (difco) and 7 Gm. of sodium chloride to a liter of distilled water. The lysate was filtered through a Coors porcelain candle having a porosity of 3. In this manner, a phage preparation having a titer of 10^8 to 10^9 plaque-forming particles per milliliter was obtained without difficulty. It characteristically produces plaques which are quite clear and regular in outline, and which are about 1.5 mm. in diameter. If desired, this material may be used without dilution, but it is convenient to dilute the stock phage 1:10 to 1:100 before employing it in the tests. The phage is stable for an indefinite period if kept under refrigeration, but the addition of 1 per cent of chloroform aids in its preservation and does not reduce the phage titer even over long periods of storage.

The cultures which were tested were grown for a period of four to six hours in nutrient broth adjusted to a pH of 7.0. When a light turbidity was evident, one loopful of the broth culture was spread over an area, having a diameter of about 20 mm., on the surface of a plate of solid medium. Seven to nine cultures were tested on a single plate.

From the Communicable Disease Center, Public Health Service, United States Department of Health, Education, and Welfare, Atlanta, Ga.
Received for publication April 5, 1954.

When the plates with the covers partially removed had become thoroughly dry, a drop of the "O-1" phage was placed in the center of each inoculated area by means of a dropper delivering about 0.03 ml. The plates were again allowed to dry and were incubated at 37° C. for a period of six to eighteen hours. At this time they were examined for evidence of lysis.

The loops which were used to spread the cultures were similar to those described by Craigie and Yen[3] for use in the phage typing of *S. typhosa.*

Droppers* should deliver the desired amount of phage when held in an upright position. They should not be allowed to come in contact with the plate during the application of the phage. With reasonable precautions in the handling of the phage, no difficulty was experienced in maintaining it free of contamination for a period of weeks.

The choice of media on which the tests are to be performed depends upon the object of the investigation. If it is desired to detect Salmonella which have been isolated on brilliant green agar,[4] colonies should be fished directly from this medium to nutrient broth. They are replated on brilliant green agar (Difco) and tested for susceptibility to the "O-1" phage by the procedure outlined previously. The typical reaction was that of alkali production in the area of growth of the Salmonella and complete lysis of the culture within the area of phage application. An occasional culture either may be lysed partially or the lysed area may be overgrown to some degree by resistant organisms. The use of a hand lens for the examination of such tests is helpful. Bacteria which ferment rapidly either lactose or sucrose will produce an acid reaction recognized by the development of a yellow color by the phenol red indicator. Due regard must be allowed for the fact that *S. typhosa* and *S. paratyphi C* will not grow on this medium. The latter serotype is rarely found in the United States, however. Occasional strains of other serotypes, such as *S. gallinarum* and *S. pullorum,* will fail to grow. If the presence of *S. typhosa* is suspected, all cultures which fail to grow on brilliant green must be retested on MacConkey's agar using the Vi phages (I-IV pool) of Craigie and Yen. This method allows the specific identification of this serotype. Most strains of a few phage types of *S. typhosa* have proved resistant to the action of the "O-1" phage.[5]

In this laboratory a modified phenol red medium has been used successfully for testing the phage susceptibility of enteric bacteria. The medium† has the following composition:

Bacto-Yeast Extract	3	Gm.
Proteose Peptone, Difco	10	Gm.
Sodium Chloride	5	Gm.
Bacto-Lactose	10	Gm.
Saccharose, Difco	10	Gm.
Bacto-Phenol Red	0.08	Gm.
Sodium Desoxycholate, Difco	1	Gm.
Bacto-Agar	20	Gm.

The medium should have a pH of 6.9 to 7.0 without further adjustment.

It gave results comparable with those obtained on brilliant green agar and, with a very few exceptions, allowed unrestricted growth of all organisms of the family *Enterobacteriaceae.* The elimination of the need for retesting, which occurs with cultures which fail to grow on brilliant green, is an obvious advantage. If rapid and specific recognition of *S. typhosa* is an objective, parallel tests of all colonies with "O-1" and with the I-IV mixture of Vi phages is feasible.

RESULTS

A comparison of the results of phage tests and of tests with polyvalent Salmonella serum as applied to 542 consecutive cultures received in this laboratory for routine diagnostic study is shown in Table I. It is evident that

*The droppers used to deliver the phage were selected from stock sold by the A. H. Thomas Company as No. 2248-F, plastic caps and dropper assembly.

†Trade names are used as a means of identifying the product and their use does not constitute endorsement by the United States Public Health Service.

there is little difference in the two methods in respect to the number of false results obtained, for the polyvalent serum is less likely to give false negative reactions with a Salmonella than is the phage while it is more likely to give a false positive reaction with a non-Salmonella than is the phage. Only about 2.6 per cent of the Salmonella in this series failed to grow on brilliant green agar.

TABLE I. COMPARISON OF SPECIFICITY OF SERUM AND PHAGE FOR THE IDENTIFICATION OF SALMONELLA

CULTURES TESTED	REACTION WITH "O-1" PHAGE		REACTION WITH POLYVALENT SERUM	
	NO. LYSED	NO. NOT LYSED	NO. AGGLUTINATED	NO. NOT AGGLUTINATED
Salmonella				
Growing on brilliant green	406	10	413	3
Not growing on brilliant green†	9	2	11	0
Total	415	12	424	3
Non-Salmonella*				
Growing on brilliant green	3	64	24	43
Not growing on brilliant green†	1	47	8	40
Total	4	111	32	83

*These cultures were submitted as enteric pathogens for routine laboratory diagnosis.
†Retested on MacConkey's medium.

The susceptibility of the Enterobacteriaceae to the "O-1" phage is shown in Table II. Except as indicated, all cultures were tested on the modified phenol red medium. The Bethesda group of paracolons,[6] which are often confused with Salmonella, were uniformly resistant. It is interesting to note that all diphasic Arizona paracolons[7, 8] are susceptible, while only a small percentage of the monophasic Arizona types[9] are attacked by the phage. The extremely close relationships between the diphasic Arizona cultures and the Salmonella as exemplified by their serologic characteristics, and as demonstrated by their reaction to the "O-1" phage is worthy of note. Although the antigent mosaic of the monophasic Arizona types also merges into that of the Salmonella, the resistance of this group to the phage is striking by comparison. In fact, nine of the ten cultures which were lysed by the "O-1" phage (Table II) may actually represent a single original strain, since the antigenic formula of all was identical (O and H), and all were received from the same geographic area.

Cultures representative of many serologic types of *Escherichia coli* and other cultures showing strong serologic relationships to the Salmonella are included in the ninety-three cultures listed as Escherichia in Table II. Only one culture, an O55:B5, was lysed by the "O-1" phage and then on brilliant green agar only. One culture described by Ewing and Hucks[10] as a provisional Shigella type was susceptible to the phage. Other Shigella representing all of the recognized types were negative. Salmonella of all somatic groups, with one exception, have proved susceptible to phage action. Thirteen cultures representing three serotypes of Salmonella having a common O antigen (XXXV) resisted lysis by the phage. These cultures were selected for special

study and are not included in Tables I or II since no representative of this somatic group was encountered among the 427 consecutive, routine cultures sent to this laboratory. It is tempting to speculate that phage resistance on the part of this group may be correlated with the presence of a K antigen (B4) commonly occurring in certain cultures of *E. coli* and in serotypes of Salmonella O group XXXV as reported by Olarte and Varela.[11] An analysis of the twelve Salmonella cultures whose resistance to the "O-1" phage is indicated in Table I shows that they consist of six serotypes, as follows: *S. derby* (4); *S. anatum* (2); *S. newington* (2); *S. pullorum* (2); *S. tennessee* (1); and *S. senftenberg* (1). Examination of additional cultures (not included in Tables I and II) of the previously mentioned types and of other types belonging to these O groups revealed a high percentage of phage resistance among those organisms classified in Group E of the Kauffmann-White schema. Such cultures possess somatic antigen III,X: III,XV: or I,III,XIX. About 300 cultures of Salmonella submitted for reference diagnosis and not included in this report have been tested for susceptibility to the "O-1" phage. Among this group and also that reported on in the present paper, approximately one-half of the cultures which were found to be resistant to phage belonged to somatic group E. At the present time no explanation can be offered for these facts. Within the limitations imposed by the choice of indicator strains used, lysogenicity did not appear to be a significant factor.

TABLE II. SUSCEPTIBILITY OF THE ENTEROBACTERIACEAE TO THE O-1 PHAGE

BACTERIAL GROUPS	NO CULTURES TESTED	NO. LYSED BY PHAGE	PER CENT LYSED BY PHAGE
Salmonella*	427	415	97.0
Shigella	195	1†	0.5
Proteus	9	0	0.0
Serratia	3	0	0.0
Escherichia	93	1	1.1
Klebsiella	7	0	0.0
Aerobacter	7	0	0.0
Paracolons:			
Bethesda	195	0	0.0
Arizona:			
Monophasic	162	10	6 2
Diphasic	29	29	100.0
Miscellaneous	95	1	1.1

*Tested on Difco brilliant green agar. Repeated on MacConkey's agar when growth failed. All other cultures tested on modified phenol red agar.

†This culture was described as a provisional type.

DISCUSSION

The use of concentrated phage for generic identification of a bacterial genus is a departure from the usual methods of application of this agent to the separation of bacterial groups. Specificity is not dependent, within wide limits, upon adjustment of phage dilutions. The phage typing procedure for *S. typhosa* and for *S. paratyphi B* as well as the techniques used for delineation of certain Salmonella O groups are dependent upon careful adjustment of phage dilution to impose the desired degree of specificity. On the other hand, susceptibility of Salmonella to highly concentrated "O-1" phage seems to be a

function of some basic genetic or physiologic characteristics which are largely confined to Salmonella and to the closely related diphasic Arizona paracolons. The only common meeting ground for these two groups which suggests itself at the present time is the possibility of mediation of susceptibility by the presence of identical or similar rough antigens. Certainly, many rough Salmonella cultures in which no O antigens can be recognized are susceptible to lysis by the "O-1" phage.

SUMMARY

A rapid, inexpensive, simple, and specific technique for the identification of the genus Salmonella by means of a bacteriophage is described. Such a procedure should be applicable to the screening of Salmonella during surveys to determine the incidence of enteric pathogens or in other circumstances where large numbers of colonies from isolation plates must be processed. The uniform susceptibility of the diphasic Arizona paracolons to the "O-1" phage constitutes a diagnostic tool by means of which the ecology and the pathogenicity of this group may be clarified. The cost of materials used in phage tests is approximately one-tenth that of agglutination tests employing polyvalent Salmonella serum and the two methods are of comparable although inverse specificity. From 200 to 400 cultures can be tested with ease by one person during a single work day.

We wish to thank Dr. A. Felix of the International Reference Laboratory for Enteric Phage Typing for supplying the original specimen of phage used in this work. We also express our appreciation to Dr. W. H. Ewing and Miss Mary G. West of the Communicable Disease Center, Chamblee, Georgia, for counsel and for supplying many of the cultures used in these experiments.

REFERENCES

1. Edwards, P. R., and Ewing; W. H.: A Manual for Enteric Bacteriology, Chamblee, Ga., 1951, Department of Health, Education, and Welfare, Public Health Service, Communicable Disease Center.
2. Felix, A., and Callow, Bessie R.: Typing of Paratyphoid B Bacilli by Means of Vi Bacteriophage, Brit. M. J. **2**: 127-130, 1943.
3. Craigie, J., and Yen, C. H.: The Demonstration of Types of *B. typhosus* by Means of Prepartions of Type II Vi Phage, Canad. J. Pub. Health **29**: 448-463, 1938.
4. Kristensen, M., Lester, V., and Jurgens, A.: On the Use of Trypsinized Casein, Bromthymol blue, Brom-cresol Purple, Phenol Red and Brilliant Green for Bacteriological Nutrient Media, Brit. J. Exper. Path. **6**: 291-299, 1925.
5. Felix, A., and Anderson, E. S.: Bacteriophage, Virulence and Agglutination Tests With a strain of *Salmonella typhi* of Low Virulence, J. Hyg. **49**: 350-364, 1951.
6. Edwards, P. R., West, Mary G., and Bruner, D. W.: Antigenic Studies of a Group of Paracolon Bacteria (Bethesda Group), J. Bact. **55**: 711-719, 1948.
7. West, Mary G., Edwards, P. R., and Bruner, D. W.: A Group of Diphasic Paracolon Bacteria, J. Infect. Dis. **81**: 24-27, 1947.
8. Edwards, P. R., and West, Mary G.: Unusual Types of Enteric Bacteria, J. Infect. Dis. **87**: 184-188, 1950.
9. Edwards, P. R., West, Mary G., and Bruner, D. W.: The Serologic Classification of the Arizona Group of Paracolon Bacteria, J. Infect. Dis. **81**: 19-23, 1947.
10. Ewing, W. H., and Hucks, M. C.: Four New Provisional Serotypes of *Shigella*, J. Bact. **69**: 575-580, 1952.
11. Olarte, J., and Varela, G.: A Complete Somatic Antigen Common to *Salmonella adelaide, Escherichia coli*—Gomez and*Escherichia coli* 0111:B4, J. Lab. & Clin. Med. **40**: 252-254, 1952.

INFLUENCE OF REPEATED HISTOPLASMIN SKIN TESTS ON PRECIPITINS AND COMPLEMENT-FIXING ANTIBODIES

S. B. Salvin, Ph.D.,* R. W. Weber, M.D.,** D. B. Lackman, Ph.D.,*
Jane Nishio, M.S.,* and Gladys Menges, R.N.***
Hamilton, Montana, and Kansas City, Kan.

HISTOPLASMOSIS in human beings has been shown to give rise to homologous precipitating and/or complement-fixing antibodies.[1-4] These antibodies have at times been transitory, especially in mild or acute cases; at other times, they have been detected over an extended period. Complement-fixing antibodies, for example, have persisted in some cases as long as four to five years from the time of onset of disease.[5]

Since patients are frequently skin tested shortly before serum is drawn for serologic assay, the possibility exists that in such cases, the presence of humoral antibodies may be an expression of booster response in a sensitive individual. Recently, twenty-two normal volunteers were skin tested weekly for seven weeks with 0.1 ml. of a 1:100 dilution of histoplasmin (lot H-40).[6] No significant change in skin reactivity was noted during the period of study. Ten of fifteen patients who reacted to histoplasmin, however, showed a change in humoral antibodies. Six of the positive reactors developed significant titers in the collodion-agglutination test between the third and sixth week after the initial skin test. Of the four other skin reactors, all showed transient reactions. The conclusion was drawn that repeated weekly histoplasmin skin tests may result in positive collodion-agglutination reactions in skin-test-positive individuals.

Another group of twenty-five healthy volunteer medical students, fourteen of whom were skin-test positive, received six weekly intradermal injections with 0.1 ml. of lot H-42 histoplasmin (1:100).[7] Fifty-seven per cent of the fourteen histoplasmin-positive students exhibited a rise in antibody as measured by the collodion-agglutination test. When the complement-fixation test was used, sera of 79 per cent of the skin-sensitive students showed a rise in antibody with a soluble supernate of the ground cells of the yeast phase as antigen, and 86 per cent with histoplasmin as antigen.

Since the value of some serologic results might be questioned because of possible booster effects, it seemed advisable to determine the extent to which such effects might occur. Accordingly, thirty-two healthy human volunteers were skin tested with histoplasmin five times over a period of eight weeks, and several serologic methods were employed to test the effect of this procedure on the level of antibody. Results of testing sera from these volunteers by two complement-fixation techniques (each with three different antigens) and by one precipitation technique are presented in this paper.

Received for publication March 25, 1954.

*From the United States Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, National Microbiological Institute, Rocky Mountain Laboratory, Hamilton, Montana.

**University of Kansas Medical Center, Kansas City, Kan.

***Midwestern Communicable Disease Center, Kansas City, Kan.

MATERIALS AND METHODS

Thirty-two medical students, most of whom were residents of Kansas, were accepted at random for study. Blood was drawn from each of the individuals before the intradermal skin tests. One tenth milliliter of a 1:100 dilution of histoplasmin (H-42) was employed with fresh dilutions made each week prior to actual use. The patients were skin tested five times and bled at intervals over a period of eight weeks (as indicated in Table I).

Precipitation tests were carried out with broth filtrate from the mycelian phase cultures as antigen. Undiluted serum and dilutions of antigen were drawn into three-inch lengths of capillary tubing, incubated for two hours at 37° C., and refrigerated for thirty-six hours at 3° to 5° C. A control of uninoculated asparagine-glucose medium was included in each test.[1]

The complement-fixation tests were carried out by both 100 per cent and 50 per cent end-point techniques. In the former technique, a Bengtson-type test[2, 8] was used with two full units of complement, two units of hemolysin, two one-hour fixations at 37° C., and that amount of antigen which gave the highest complement-fixation titer with a serum of known capacity. In the second type, the test of Kent and Rein,[9] as performed by Saslaw and Campbell,[10] was used, with three 50 per cent units of complement, and overnight fixation at 3° to 6° C. between the primary and secondary fixations (AMS type). An optimal antigen dilution was also used in this test.

Three antigens were used in the complement-fixation tests. a. Whole cells of the yeast phase, grown in broth* for five days at 37° C., were killed by exposure to 56° C. for one hour, washed, and resuspended in saline solution.[1] b. Whole cells, grown in the previously mentioned broth were killed by exposure to 1:10,000 Merthiolate for five days at 37° C. The organisms were then ground at 3° to 5° C.; the mixture was centrifuged, and the clear supernate used as antigen. One lot of antigen was ground by hand[10]; a second lot was ground in a Mickle tissue disintegrator. c. The broth filtrate from mycelian growth was prepared by growing the mycelium on the surface of an asparagine-glucose broth, as described by Smith.[11]

RESULTS

Fourteen of the thirty-two medical students showed no skin reactivity at any time during the course of these experiments. With one exception, none of the fourteen skin-negative individuals showed antibodies against any of the antigens in the serologic tests employed in this study. That exception was one individual (T. M.), whose serum on the fourth week had a 1:5 titer in the presence of soluble yeast-phase antigen in the complement-fixation test, in which three 50 per cent units of complement were employed.

Eighteen of the thirty-two medical students showed a skin-test reaction to histoplasmin after the first bleeding. These reactions varied from very weakly positive, as shown by induration diameters of 5 to 9 mm., to those which showed a very marked and extensive area of induration of up to 20 to 25 mm. in diameter. After five skin tests within an eight-week period, none of the individuals showed a marked increase in transverse diameter of the induration area. Several patients had brief fluctuations in the degree of skin reactivity,

*Difco casamino acids 10.0 Gm.
Dextrose 3.0
Yeast-extract dialysate, equivalent of 3.0
Sodium chloride 2.5
Cysteine hydrochloride 0.5
Potassium chloride 2.5
Disodium phosphate 4.0
Distilled water to make 1,000 ml.

although no trend toward increase or decrease of induration area was evident in any individual. It seemed, therefore, that repeated injections of histoplasmin did not influence the size of the skin reaction in skin-positive individuals.

TABLE I. RESULTS OF COMPLEMENT-FIXATION TESTS WITH THREE 50 PER CENT UNITS OF COMPLEMENT AND OVERNIGHT FIXATION AT 3° TO 6° C. (AMS-TYPE TEST) ON SERA FROM THOSE PATIENTS WHO HAD POSITIVE SKIN TESTS

SUBJECT	ANTIGEN	TIME (IN WEEKS)					
		1	2	3	4	6	8
D. A.	SYP	AC	20	ND	20	5	20
	H	AC	20	ND	10	10	10
	WYC	AC	10	ND	10	5	10
M. I. A.	SYP	0	0	0	0	5	0
	H	0	5	10	10	10	5
	WYC	0	0	0	0	0	0
H. A.	SYP	0	0	0	40	20	0
	H	0	0	0	20	10	0
	WYC	0	0	0	10	10	0
R. B.	SYP	0	0	10	10	10	10
	H	0	5	20	10	20	10
	WYC	0	0	5	0	5	5
B. B.	SYP	0	0	0	0	0	0
	H	0	0	5	0	0	0
	WYC	0	0	0	0	0	0
W. C.	SYP	AC	0	10	10	10	ND
	H	AC	5	10	10	10	ND
	WYC	AC	5	0	0	5	ND
V. E.	SYP	AC	20	20	20	10	10
	H	AC	0	10	10	10	5
	WYC	AC	0	0	0	5	0
K. H.	SYP	AC	0	ND	0	ND	5
	H	AC	0	ND	0	ND	0
	WYC	AC	0	ND	0	ND	0
G. E. S.	SYP	0	40	20	20	20	20
	H	0	5	40	40	40	40
	WYC	0	0	0	0	0	0
R. W.	SYP	AC	0	0	0	0	5
	H	AC	0	20	20	10	10
	WYC	AC	0	0	5	5	5

In Tables I and II, SYP : Soluble supernate from ground cells of the yeast phase.
H : Broth filtrate from the growth of the mycelium asparagine broth (histoplasmin).
WYC : Whole cells of the yeast phase.
AC : Anticomplementary serum.
ND : Not enough serum available to conduct test.
"0" indicates a titer of less than 1:5.

None of the skin-test-positive patients had precipitins evident in the sera from the first bleeding. Two subsequently did show a change in the precipitation reaction, however. When the sera of the first of these two patients were examined by optimal-proportion techniques, slight precipitates developed in the capillaries with serum dilutions of 1:2 and undiluted during the third and fourth weeks, respectively. When the sera of the other patient were examined by optimal-proportion techniques, slight precipitates were present in serum dilutions of 1:2, 1:2, and undiluted during the fourth, fifth, and sixth bleedings,

respectively. It is to be emphasized that these precipitates, even at the point of optimum dilutions, did not approach in size or opacity those seen with sera in proved or probable active cases of histoplasmosis. These sera, therefore, would not be considered "positive," but merely "doubtful."

TABLE II. RESULTS OF COMPLEMENT-FIXATION TESTS WITH TWO 100 PER CENT UNITS OF COMPLEMENT (BENGTSON-TYPE TEST) ON SERA FROM THOSE PATIENTS WHO HAD POSITIVE SKIN TESTS

SUBJECT	ANTIGEN	TIME (IN WEEKS)					
		1	2	3	4	6	8
D. A.	SYP	0*	2	ND	0 (at 1:4)	0	0
	H	0	ND	ND	ND	4	4
	WYC	0	0	ND	0	0	0
H. A.	SYP	0	0	ND	ND	0	0
	H	0	ND	ND	ND	4	4
	WYC	0	0	0	0	0	0
M. L. A.	SYP	0	0	0	0	0	0
	H	ND	4	4	4	4	4
	WYC	0	0	0	0	0	0
R. B.	SYP	0	ND	0	0	0	0
	H	ND	ND	4	ND	8	4
	WYC	0	0	0	0	0	0
B. B.	SYP	0	0	0	0	0	0
	H	ND	4	8	4	ND	ND
	WYC	0	0	0	0	0	0
W. C.	SYP	ND	8	4	ND	4	ND
	H	ND	8	ND	ND	4	ND
	WYC	0	0	0	0	0	0
V. E.	SYP	AC	0	2	2	ND	2
	H	ND	ND	ND	ND	ND	4
	WYC	0	0	0	0	0	0
K. H.	SYP	2	0	ND	0	ND	2
	H	ND	ND	ND	ND	ND	4
	WYC	0	0	0	0	0	0
G. E. S.	SYP	AC	0	4	4	2	ND
	H	ND	ND	ND	4	4	ND
	WYC	0	0	0	0	0	0
R. W.	SYP	0	0	0	0	0	0
	H	ND	0	4	AC	ND	4
	WYC	0	0	0	0	0	0

For key to abbreviation see Table I.
*"0" indicates titers of less than 1:2.

Fixation of complement was essentially negative in sera from the first bleeding. Results in the complement-fixation tests with the sera subsequently drawn depended on the nature of the antigen and on the type of test. Histoplasmin and the soluble supernate from ground cells of the yeast-phase-fixed complement with some sera from the skin-positive individuals after they had been repeatedly skin tested. This demonstration of antibodies by the two soluble antigens occurred in both types of complement-fixation tests, although the titers were much higher in the test employing three 50 per cent units of complement (AMS-type test). In the AMS-type test (Table I), sera from nine

of the eighteen skin-positive students showed antibodies on at least one bleeding date in the presence of the soluble supernate from ground cells of the yeast phase as antigen, sera from nine showed antibodies with histoplasmin as antigen, and sera from six showed antibodies with whole yeast cells as antigen. The antibodies were of a lower titer and of shorter duration with the whole cells as antigen than with either the soluble supernate or histoplasmin. That antibodies were detectable in sera for shorter periods of time when whole cells were used as the complement-fixing antigen is indicated by the fact that of the sera drawn after the students had been skin tested at least once, 26 of 51 (51 per cent) were positive with the soluble supernate from ground yeast cells as antigen, 34 of 51 (67 per cent) were positive with histoplasmin as antigen, and only 15 of 51 (29 per cent) with whole yeast cells as antigen.

In the Bengtson-type test (Table II), with histoplasmin as antigen, ten individuals who had been skin tested repeatedly had at least one positive serum; with the soluble antigen from ground cells of the yeast phase as antigen, five individuals had positive sera. These titers were generally lower and less frequent than those seen in the more sensitive AMS-type test (Table I). In this Bengtson-type test with whole cells of the yeast phase as antigen, however, not one of the eighteen sera from the skin-positive individuals who had been repeatedly skin tested showed the development of complement-fixing antibodies.

DISCUSSION

Histoplasmin is now used extensively as a skin-testing antigen in patients suspected of having histoplasmosis. Frequently, after a positive skin test has developed, serum is drawn and assayed for antibodies against *Histoplasma capsulatum*. It is therefore essential to know whether reactions in a given serologic test are altered by skin testing with histoplasmin. Obviously, the ideal situation would involve bleeding the patient before a skin test is performed. The fact that the histoplasmin skin test is a good tool for differentiating those patients who have or have had histoplasmosis from those who have not, however, may lead some investigators to skin test a patient before drawing blood for serologic assay.

After only one skin test, seemingly healthy skin-positive individuals have been shown not to yield antibodies demonstrable by collodion-agglutination or by a complement-fixation technique with a soluble extract from the yeast phase as antigen.[6, 7] When a sensitized patient's serum is tested after two or more skin tests, however, antibodies have been demonstrated by two different techniques: a. by collodion-agglutination test with histoplasmin adsorbed onto collodion particles;[6] and b. by a complement-fixation test in which three 50 per cent hemolytic units of complement were used with overnight fixation and in which either histoplasmin or soluble supernate from ground cells of the yeast phase were employed as antigen.[7] Is this booster response so pronounced that it can be expected to mar the significance of serologic testing of serum drawn after a patient has been skin tested? Or is this a response that is more likely to occur with one particular test or antigen than another?

The results reported in this paper indicate that the test and the antigen are of great importance in determining antibody production after two or more skin tests. For example, the more highly sensitive of the complement-fixation tests with histoplasmin or the soluble supernate from ground cells of the yeast phase as antigen tended to show antibodies after a sensitized individual had been skin tested several times. When whole cells of the yeast phase were used in this same highly sensitive complement-fixation test, the number of individuals with positive titers, as well as the amount of demonstrable antibody in the positive individuals, was less. When a slightly less sensitive test was used with the whole yeast cell as antigen, none of the eighteen skin-test-positive individuals who had been skin tested five times showed complement-fixing antibodies. Apparently, then, the booster response in sera from patients who have been skin tested several times is more pronounced to one type of antigen, i.e., the one that is contained in the histoplasmin used in skin testing.

The precipitin test did not show distinctly positive reactions in the foregoing studies. This is in contrast to the collodion-agglutination test,[6, 7] which has been described as showing humoral antibodies in 18 of 29 skin-positive patients (62 per cent) after these individuals had been skin tested several times with histoplasmin.

It is to be emphasized that the studies described in this paper were conducted on apparently healthy individuals. Therefore, the possibility should be borne in mind that patients in the acute or convalescent phases of histoplasmosis may show a greater response than normal individuals. It is accordingly recommended that if possible, blood be drawn prior to skin testing. Also, a serologic test should be used which would tend not to show antibodies in healthy skin-positive individuals who had been repeatedly skin tested. Such tests seem to include a Bengtson-type complement-fixation test with whole cells of the yeast phase as antigen, or a precipitation test with broth filtrate from the mycelian growth as antigen.[1]

SUMMARY

Repeated positive histoplasmin skin tests in normal medical students did not show increases in antibody titers when whole cells of the yeast phase were used as antigen in a Bengtson-type complement-fixation test or when a soluble supernate from the growth of the mycelian phase in broth was used as antigen in a precipitation test. A booster response, however, as evidenced by increased antibody titers, was apparent when histoplasmin or the supernate from ground cells of the yeast phase was used as complement-fixing antigen.

REFERENCES

1. Salvin, S. B., and Furcolow, M. L.: Precipitins in Human Histoplasmosis, J. Lab. & Clin. Med. **43**: 259-274, 1954.
2. Salvin, S. B.: Complement-Fixation Studies in Experimental Histoplasmosis, Proc. Soc. Exper. Biol. & Med. **66**: 342-345, 1947.
3. Grayston, T. J.: A Study of the Complement-Fixation Reaction in Histoplasmosis, J. Lab. & Clin. Med. **40**: 90-101, 1952.
4. Furcolow, M. L., Bunnell, I. L., and Tenenberg, D. J.: A Complement-Fixation Test for Histoplasmosis. II. Preliminary Results With Human Sera, Pub. Health Rep. **63**: 169-178, 1948.

5. Salvin, S. B.: Unpublished notes.
6. Prior, J. A., and Saslaw, S.: Effect of Repeated Histoplasmin Skin Tests on Skin Reactivity and Collodion Agglutination, Am. Rev. Tubere. **66**: 588-593, 1952.
7. Saslaw, S., and Campbell, C. C.: Effect of Histoplasmin Skin Testing on Serologic Results, Proc. Soc. Exper. Biol. & Med. **82**: 689-691, 1953.
8. Bengtson, I. A.: Complement-Fixation in the Rickettsial Diseases—Technique of the Test, Pub. Health Rep. **59**: 402-405, 1944.
9. Kent, J. F., and Rein, C. R.: Serodiagnosis of Amebiasis, Science **103**: 598, 1946.
10. Saslaw, S., and Campbell, C. C.: The Use of Yeast Phase Antigens in a Complement-Fixation Test for Histoplasmosis, J. LAB. & CLIN. MED. **33**: 811-818, 1948.
11. Smith, C. E.: Coccidioidomycosis, M. Clin. North America **27**: 790-807, 1943.

DETERMINATION OF IRON IN SMALL AMOUNTS OF SERUM AND WHOLE BLOOD WITH THE USE OF THIOCYANATE

HUGH W. JOSEPHS, M.D.
BALTIMORE, MD.

METHODS employing thiocyanate for the determination of iron have been little used in recent years, especially in this country. About fifteen years ago, the methods then in use were criticized by a number of investigators.[1-4] There appears to be no doubt that the final color is subject to modification by changes in acidity, presence of phosphates, and possibly other substances; but many of the difficulties had already been successfully met and others were not insuperable.[3, 5, 6, 7] In spite of the difficulties, which are real, the use of thiocyanate has a number of advantages,[8] especially that the intensity of the color extracted in a proper organic solvent is such as to permit an accurate determination on considerably less material than is required by other methods. [2, 4, 9, 10] For the determination of serum iron in infants and children this advantage was felt to be great enough to outweigh the disadvantages. Accordingly, a method using thiocyanate has been worked out which has been in satisfactory use for about two years.

METHOD FOR SERUM

In general the procedure is the following: the serum iron complexes are broken up by acid digestion; proteins are precipitated with trichloroacetic acid; after centrifugation the iron in the supernatant is oxidized to the ferric state and determined colorimetrically as ferric thiocyanate in an ethyl acetate extract.

The details of the method are as follows:

A. *Preparation of Reagents.*—

1. *Hydrochloric acid:* This is prepared by distillation from concentrated HCl, then made up to approximately 0.2 N.

2. *Trichloroacetic acid:* This is purified by vacuum distillation, then made up to a 25 per cent solution with iron-free distilled water.

3. *Ammonium thiocyanate:* This is made up to 30 per cent. It is then purified by repeated extraction with 20 per cent of its volume of ethyl acetate. Three extractions should be sufficient. A slight color in the ethyl acetate will persist on further extraction. This may be disregarded.

4. *Distilled water:* If it is found to contain iron it should be redistilled. To test, place 2 ml. in an iron-free glass-stoppered cylinder, add 1 drop of 20 per cent HNO_3, heat in a boiling water bath for ten minutes, and add 0.3 ml. thiocyanate reagent and 0.5 ml. ethyl acetate. Shake and allow to separate. If there is a recognizable pink shade in the ethyl acetate, there is too much iron.

From the Department of Pediatrics, The Johns Hopkins University School of Medicine, and the Harriet Lane Home of the Johns Hopkins Hospital.

Received for publication, Sept. 12, 1953.

5. *Iron standard:* Dissolve iron wire (for analysis) in an excess of sulfuric acid to which a small amount of nitric acid has been added. The stock solution should be made up to contain 2 mg. per milliliter. In making up this stock, ordinary distilled water may be used. From this a second stock is prepared to contain 50 μg per milliliter from which a more dilute working solution containing 5 to 10 μg per milliliter is prepared on the day on which it is to be used. The solution containing 50 μg per milliliter can be kept for about a month.

6. *Ethyl acetate* (Merck reagent and Eimer and Amend reagent): This was found to be free of iron as used. It was therefore unnecessary to purify it further.

7. For use with the serum iron method the nitric acid is diluted with an equal quantity of distilled water (iron-free). When the laboratory distilled water was heated with a drop of this 50 per cent nitric acid and tested as described previously, no color was obtained, from which it was concluded that neither the water nor the nitric acid contained sufficient iron to be appreciable. Not all samples of nitric acid will necessarily be free of all trace of iron, however. For this reason, 20 per cent nitric acid is suggested in testing the distilled water, to minimize the possible effect of iron in the acid. For use in the determination any slight iron impurity in the nitric acid will appear in the blank, and as long as the value of the blank is not excessive, one need not go to great lengths to eliminate it.

The reagents should be kept in glass-stoppered bottles protected by some form of dust cap. Pipettes should not be dipped into the stock bottles unless the pipettes are kept meticulously free of iron and thoroughly protected from dust.

Glassware.—The tubes for collection of blood should be placed in cleaning solution over night, then rinsed thoroughly with iron-free distilled water and dried free of contact with dust. They may be stoppered with clean cork stoppers. It is well to avoid contact between the stopper and the specimen, but in my experience this is not a source of error. No tube should be used of which the cleaning history is unknown. Syringes should be similarly treated unless they are found to be routinely free of iron.

Pipettes should preferably be kept separate to be used for this one method. If this can be done, they need only an initial treatment with cleaning solution. Thereafter they may be simply rinsed with distilled water after each use and kept under dust-free conditions.

In a method such as this, in which small amounts of solution have to be handled, the ordinary use of graduated transfer pipettes will not suffice. All transfers are made with capillary pipettes fitted with rubber nipples. This method does not allow for measurement and a special measuring device had to be prepared. This was done by breaking a 10 ml. transfer pipette with graduations reaching to the tip about 2 to 3 inches from the tip. The tip was then carefully sealed. This gave a 2 to 3 ml. cell graduated in tenths of a milliliter, into which fluid could be transferred, measured, and removed again to any extent desired.*

For the final preparation for colorimetry 5 ml. *glass-stoppered* graduated cylinders are used. After initial cleansing and rinsing, it is sufficient that they be rinsed after each use and kept inverted until the next use. The glass stoppers should be thoroughly rinsed just before use. They may be handled with one's fingers provided these have been scrubbed with soap and rinsed with distilled water.

The capillary pipettes used for transfers are made by drawing out ordinary glass tubing to form a capillary long enough to reach to the bottom of the tubes. A separate one should be kept for each procedure. As in the case of the pipettes they need only an initial cleaning with cleaning solution. Thereafter they may be merely rinsed after each use. If any deposit remains after rinsing, however, they should be retreated with cleaning solution.

B. *Stages of the Procedure.*—

1. After separation, measure the amount of serum to be analyzed into an iron-free Wassermann tube. It is desirable to have 1.0 to 0.8 ml., but as little as 0.5 ml. can be used

*There are other ways in which the problem of measurement might be met: (1) Capillary pipettes might be made of heavier material (to avoid breakage) and calibrated between 1 and 2 ml. so that transfer and measurement could be made in one operation. (2) Tubes of appropriate bore, of a length convenient for centrifuging, fitted with glass stoppers, and calibrated between 1 and 2 ml. could be used in place of the glass-stoppered cylinders.

with accurate results. If more than 1 ml. of serum is used, the amount of the reagents next to be added will have to be proportionately increased.

2. Add to the serum 0.4 ml. of 0.2 N HCl and heat at 70° to 80° C. for ten minutes, to liberate the iron from its combination with globulin.

3. After the acidified serum has cooled to room temperature, add to it 0.4 ml. iron-free distilled water and 0.4 ml. trichloroacetic acid and mix thoroughly. Allow to stand for about fifteen minutes with occasional mixing, then centrifuge at sufficient speed to throw down the protein precipitate in a solid mass leaving a clear supernatant.

The specified amounts of HCl and trichloroacetic acid are minimum safe amounts for use with no more than 1 ml. of serum. The 0.4 ml. of water is added to increase the amount of fluid to facilitate handling, but more should not be used since too great a dilution leads to incomplete protein precipitation. The sum of the separate amounts of serum, HCl, trichloroacetic acid, and distilled water should be recorded as the total fluid (T.F.) to be used later in the calculation of results. The same amounts of reagent can be used with smaller amounts of serum down to 0.5 ml.

The duration and speed of centrifuging should be such that the precipitate is sufficiently packed to allow the removal of a clear supernatant. Five minutes at 2,000 r.p.m. is sufficient.

4. Transfer the supernatant to a 5 ml. glass-stoppered graduated cylinder. It is not necessary to transfer all or any specified amount. The only requisites are that it be uncontaminated with any of the precipitate, and that it be measured and recorded. One reason for the use of graduated cylinders is that it is possible to use the graduations for the measurement of the supernatant, provided these have been tested and found to be accurate. Although it is not necessary to transfer all the supernatant, it is desirable to transfer as much as possible. The amount of iron present in the serum in many cases may be so small as to approach the lower limit of useful galvanometer deflection. Naturally the greater the amount of material available for colorimetry the better.

5. Next, add 1 drop of 50 per cent HNO_3 to the supernatant in each cylinder, and place the cylinders in a boiling, or near boiling, water bath for ten minutes, then cool to room temperature.

At this point, the iron transferred in the supernatant is in ferric form ready for the last step that will develop the color. From this point, the procedure may have to be varied for the type of colorimeter to be used. As given here, it has been devised for the microattachment of the Evelyn colorimeter. For use in other colorimeters the principal difference would be in the amount of ethyl acetate to be added and the transfer procedure depending on whether or not it is necessary to transfer a measured amount.

6. When the cylinders have cooled, add to each 1.2 ml. ethyl acetate, followed by 0.3 ml. ammonium thiocyanate reagent. Each cylinder should be stoppered and shaken violently for a few seconds immediately after the addition of the thiocyanate, then allowed to stand. As soon as the ethyl acetate layer has cleared,* it is ready for colorimetry. The color is relatively stable in ethyl acetate, but may fade rather rapidly in aqueous solution if the ethyl acetate is added after the thiocyanate. Although the color is relatively stable, it is probably best not to prepare more than three cylinders at a time, and to read them within ten minutes.

7. With one of the capillary pipettes, transfer sufficient of the ethyl acetate layer to fill one of the measuring cells to the first milliliter graduation. Then transfer exactly 0.9 ml. to the microcolorimeter cuvette. The accuracy of this procedure is of the utmost importance,

*The ethyl acetate layer should be clear in a minute or two. Occasionally emulsion formation may be troublesome. A slight residual cloudiness can easily be cleared by centrifugation, but if it is marked and persistent, iron will almost certainly be lost. In the majority of instances this has been found to be associated with inadequate protein precipitation. Increase in the amount of trichloroacetic acid was considered inadvisable because of its high "blank" value. It was found, however, that when the serum was heated sufficiently at step 2, even to the point of coagulation, the emulsion formation was practically eliminated. Whether this procedure leads to better precipitation or merely denatures the protein to the point where it no longer interferes with iron recovery has not been determined.

for only by this means can one ensure constancy in depth of solution in the colorimeter cuvette. The glass plungers supplied to ensure a constant depth are coated with a substance that gives off sufficient iron to interfere with the results. It is difficult to avoid transferring some of the aqueous layer along with the ethyl acetate from the cylinder to the measuring cell. As long as the amount is not sufficient to interfere with the transfer of ethyl acetate to the colorimeter cuvette, it may be disregarded.

8. The reading is made on the micro-attachment with the 540 filter and with the galvanometer set at 100 for exactly 0.9 ml. ethyl acetate, transferred from the measuring cell as described previously. Blank readings are made on a mixture of 0.4 ml. HCl, 0.4 ml. trichloroacetic acid, and 0.4 ml. iron-free distilled water treated exactly as is the unknown beginning with step 5.

Between each determination, the measuring cell and colorimeter cuvette should be rinsed with ethyl acetate. The cuvete should also be wiped out with a rolled-up ash-free filter paper, to remove any moisture left after removal of the ethyl acetate.*

C. *Calculations.*—

The following abbreviations are used for convenience:

T.F. Total fluid – (milliliters serum + HCl + trichloroacetic acid + water)

Sn Supernatant (amount transferred to cylinder in step 4 containing the iron on which the analysis is carried out).

G Galvanometer reading.

L_G Color density corresponding to galvanometer reading (2 – Log G).

L_S Color density corresponding to the iron present in mixture of serum and reagents present at the time of the protein precipitation. This is not actually determined but is calculated from L_G. ($L_S = L_G \times \frac{T.F.}{Sn}$).

$L_C = L_S$ – L value of blank.

It is assumed that all the iron, including that of the blank, present at the time of the protein precipitation is distributed in equal concentration between the supernatant removed for analysis and the material remaining behind after removal of the supernatant.† The value for the color density as determined (L_G), representing the proportion of total iron present in the supernatant, must be multiplied by the ratio of total fluid to supernatant to obtain the color density (L_S) that would have been obtained had all the iron been determined. From this is subtracted the L value for the blank. The result divided by the amount of serum gives the L value for 1 ml. of serum. In Table I are contained determinations on two samples of serum with details to illustrate the calculations. They also illustrate the effect of varying the amount of serum and the effect of heat on the initial HCl digestion. The results are given in figures for color density. Translation of this figure into micrograms of iron is accomplished by use of a factor the determination of which requires that the method be calibrated with standard iron preparations.

---

*The initial cleaning of the microcolorimeter cuvette introduces no problem. Apart from washing with soap or detergent to get a clean glass surface, it need only be extracted with ethyl acetate under the conditions in which it is to be used. The simplest procedure is to carry out a "dummy" blank determination and, after removing the ethyl acetate, wipe out the cuvette with a rolled-up ash-free filter paper. The only material with which we are concerned is that which might give an extractable color with thiocyanate; if this has been removed and the bottom of the tube is clean, no more cleansing is necessary. Even this procedure need not be repeated if the cuvette is used only for this determination and is kept under dust-free conditions, since each determination leaves the cuvette free of detectable iron and we are not concerned with undetectable iron.

†This assumption is one that is tacitly made in any method in which protein is precipitated, including other methods for iron. In earlier work, however, the possibility of a "co-precipitation" of iron was much discussed.[4, 7] Moore and associates,[3] after a study of this possibility, concluded in confirmation of the statements of others that after initial acid digestion the iron appeared quantitatively in the trichloroacetic acid filtrate. Moreover, as will be shown, calculation based on this assumption gave the same figures for iron recovery whether the iron was added before or after the precipitation of the protein.

TABLE I. CALCULATION OF COLOR DENSITY PER MILLILITER OF SERUM

SERUM Sample	ml.	HCl ml.	TCA* ml.	T.F. ml.	Sn ml.	G	L_G	L_S	L_C	$L_{ml.}$	
No.	1.0	0.5	0.4	2.2	1.40	83	.081	.128	.106	.106	
1	0.8	0.5	0.4	2.0	1.40	84	.075	.107	.085	.106	
	0.6	0.5	0.4	1.8	1.40	86_2	.067	.086	.064	.107	
	0.4	0.5	0.4	1.6	1.30	88	.053	.066	.044	.110	
Blank		0.5	0.4	-	-	95	.022	-	-	-	
No.	1.0	0.3	0.4	2.1	1.3	82^2	.084	.136	.116	.116	Serum heated with HCl
2	0.5	0.3	0.4	1.6	1.1	88	.055	.080	.060	.120	
	0.3	0.3	0.4	1.6	1.35	90	.046	.055	.035	.117	
	1.0	0.3	0.4	2.1	1.5	83_2	.081	.113	.093	.093	Serum unheated
	0.5	0.3	0.4	1.6	1.35	86	.063	.075	.055	.110	
	0.3	0.3	0.4	1.6	1.4	90_2	.046	.053	.033	.110	
Blank		0.3	0.4	-	-	95	.020	-	-	-	

*TCA refers to trichloroacetic acid. The other abbreviations are explained in the text.

TABLE II. CALIBRATION WITH STANDARD IRON, USING 0.5 ML. HCl

IRON (γ)	HCl. (ML.)	G	L_G	L_C	F_C*	IRON† CALC. (γ)
3.0	.5	46°	.337	.327	9.2	2.65
2.7	.5	46^2	.332	.322	8.4	2.6
2.5	.5	49°	.310	.300	8.3	2.43
2.0	.5	54^2	.264	.254	7.9	2.05
1.6	.5	62°	.208	.198	8.1	1.60
1.6	.5	60°	.222	.212	7.6	1.71
1.2	.5	70^2	.152	.142	8.4	1.15
.8	.5	77^2	.111	.101	7.9	.82
.8	.5	77	.113	.103	7.8	.83
.64	.5	80^2	.094	.084	7.6	.68
.5	.5	85^2	.068	.058	8.6	.47
.5	.5	84^2	.073	.063	7.9	.51
.32	.5	89^2	.048	.038	8.4	.307
.16	.5	93^3	.029	.019	8.4	.153
Blank	.5	98	.010	-	-	-
		Mean value of Conversion factor			8.07	

*F_c (Conversion factor) $= \frac{\text{micrograms Iron}}{L_c}$

†Iron calculated from L_c using mean value of conversion factor.

TABLE III. CALIBRATION WITH STANDARD IRON USING FIVE-TENTHS MILLILITER HYDROCHLORIC ACID AND FOUR-TENTHS MILLILITER T.C.A.

IRON (γ)	HCl. (ML.)	T.C.A. (ML.)	G	L_G	L_C	F_C	IRON CALC. (γ)
3.0	.5	.4	43	.367	.346	8.7	2.84
2.5	.5	.4	47^2	.323	.302	8.3	2.48
2.0	.5	.4	55	.260	.239	8.4	1.97
1.5	.5	.4	62^2	.204	.183	8.2	1.50
1.0	.5	.4	71^3	.144	.123	8.1	1.01
.8	.5	.4	76	.119	.098	8.2	.805
.6	.5	.4	80^1	.096	.075	8.0	.616
.4	.5	.4	85^1	.069	.048	8.3	.394
.2	.5	.4	89^3	.047	.026	7.7	.213
Blank	.5	.4	95^1	.021	-		

D. *Standardization.*—This consists of two parts. (1) Determination of the relation of color density to micrograms of iron under standard conditions and under the influence of changes, especially acidity, amount of aqueous solution, possible excess of trichloroacetic acid. (2) Determination of the color density-concentration relationship in the case of serum.

1. *Calibration with standard iron solutions:* A standard solution containing 10 μg of iron per milliliter was made up immediately before use. From this the desired amounts were pipetted into a mixture of 0.5 ml., 0.2 N HCl, and 0.5 ml. of iron-free distilled water contained in a series of cylinders. For this purpose a 0.2 ml. graduated pipette was used, the accuracy of which was determined to be adequate. (Exax Blue Line.) To each cylinder 1 drop of 50 per cent nitric acid was added and the procedure was continued as detailed previously from step 5. The results are contained in Table II and Fig. 1.

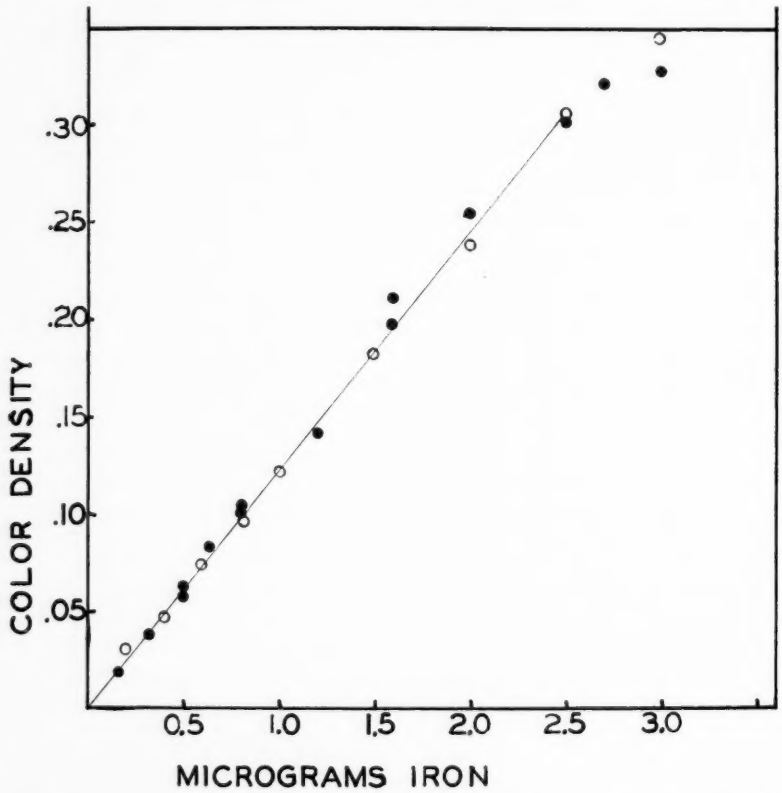

Fig. 1.—Calibration of colorimeter with standard iron. The solid circles represent standard solution made up with 0.4 ml. HCl. Those made up with HCl and trichloroacetic acid are represented by hollow circles.

Increase in the total amount of fluid by addition of distilled water made no difference in the calibration. When 0.3 ml. of *trichloroacetic acid* was included in the calibration, the results were as shown in Table III (also contained in Fig. 1). The results indicate that addition of trichloroacetic acid has caused a slight diminution in color density—about 2 per cent. It was a priori conceivable that differences in amount of protein might give rise to differences in the amount of trichloroacetic acid remaining in the supernatant and so influence the results. The smallness of the total effect makes this unlikely. Moreover, in Table I, the color density was proportional to the amount of serum from 1 ml. to 0.4 ml in the presence of the same amount of trichloroacetic acid in each instance.

2. *Calibration in the presence of serum:* This is a more complicated procedure, since there is no way of knowing a priori the amount of iron in serum. The method used was indirect and consisted in determining the color density of two samples of the same serum to one of which was added a known amount of iron. The difference between the two L_S values represents the L value for the added iron (L_{Fe}). The assumption was made that the relationship between the added iron and the corresponding L value would be the same as that for the iron originally in the serum, and therefore could be used as a factor for converting the L value for serum into micrograms of iron.

The results are contained in Table IV. It is evident that there is something in serum carried over into the supernatant that interferes with color development, as is shown by a higher conversion factor (factor by which the L value must be multiplied to obtain the figure for micrograms of iron). The over-all variation in this factor in normal cases, from which an average was computed, is considerable (8.7 to 9.6), but it must be remembered that this result involves the subtraction of two values, each one of which has an error, so that there would be expected a considerable error in the difference. For this reason it is felt that the rather wide range of values is not excessive and that the average can be used as a factor to convert figures for color density to those for micrograms of iron in the case of serum. From Table IV the average conversion factor was found to be 9.1.

TABLE IV. DETERMINATION OF FACTOR FOR CONVERTING VALUE FOR COLOR DENSITY TO MICROGRAMS OF IRON IN CASE OF SERUM

	SERUM (ML.)	IRON ADDED (γ)	T.F. (ML.)	Sn (ML.)	G	L_G	L_S	L_{Fe}*	CONVERSION FACTOR
1	.80	0	2.0	1.35	84[2]	.073	.108		
	.80	1	2.1	1.60	68	.170	.223	.115	8.7
2	.70	0	2.3	1.80	84	.076	.097		
		1	2.4	2.00	67	.173	.211	.114	8.7
3	.70	0	2.0	1.65	86	.066	.084		
		1	2.1	1.60	71[2]	.146	.192	.108	9.3
		1	2.2	1.65	71	.149	.199	.110	9.1
4	.70	0	2.1	1.65	85	.071	.091		
		1	2.2	1.70	67	.174	.224	.116	8.5
		1	2.4	1.80	68[2]	.164	.219	.107	9.4
5	.60	0	2.1	1.65	83	.081	.104		
	.60	1	2.1	1.60	69	.158	.208	.104	9.6
	.60	1	2.1	1.60	67[2]	.171	.224	.110	9.1
	.60	1	2.2	1.60	68	.169	.232	.112	8.9
6	.70	0	2.0	1.50	86	.066	.088		
	.70	1	2.1	1.65	68[3]	.162	.202	.114	8.8
	.70	1	2.1	1.60	70	.155	.204	.106	9.4
	.70	1	2.2	1.80	67[1]	.172	.210	.107	9.3
7	.65	0	1.95	1.45	82	.086	.120		
	.65	1	2.05	1.50	67[3]	.169	.231	.111	9.0
8	.70	0	2.1	1.50	83[2]	.079	.111		
		1	2.1	1.60	67[2]	.171	.224	.113	8.8
		1	2.2	1.60	67	.174	.239	.111	9.0
9	.50	0	1.7	1.45	83[2]	.078	.092		
	.50	0	1.7	1.40	85[1]	.069	.084		
	.50	1	1.7	1.40	91	.150	.186	.104	9.6
	.50	1	1.7	1.35	69	.161	.203	.110	9.1
	.50	1	1.8	1.40	69	.161	.207	.109	9.2
	.50	1	1.7	1.40	69	.161	.197	1.04	9.6
	.50	1	1.7	1.35	69[2]	.158	.204	1.11	9.0
	.50	1	1.7	1.30	69[2]	.158	.207	1.14	8.8

*The apparent discrepancies that appear in many of the figures for L_{Fe} are due to the fact that the amount of HCl used for the initial digestion was varied. This caused an increase in the blank value that had to be taken into account in the determination.

It was stated in the last paragraph that there was something in serum that interfered with color development. Actually the possibility should be excluded that iron was not precipitated with the proteins and so kept from reaching the supernatant. This was accomplished by adding iron to the supernatant at the end of step 4 and determining its conversion factor. The factor was found to be the same as that determined for iron added before protein precipitation. From this it may be concluded that the added iron was not precipitated with the proteins.

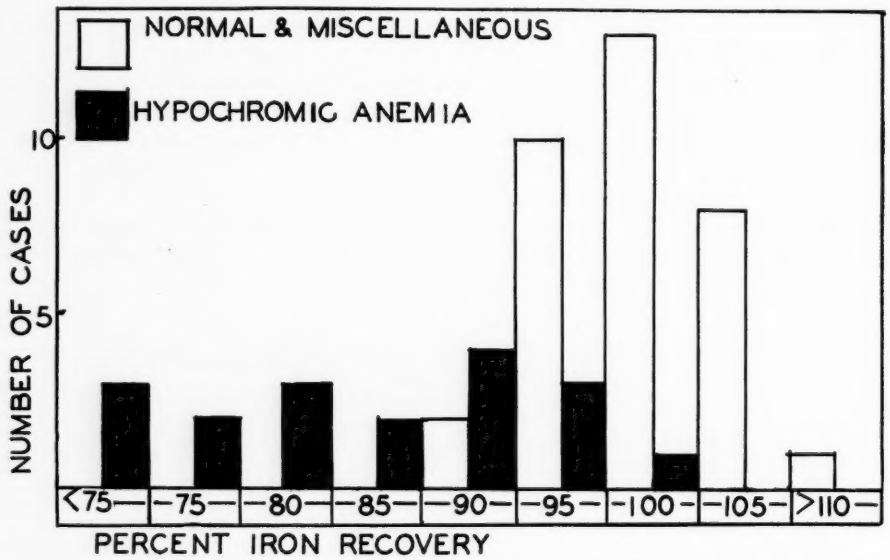

Fig. 2.—Percentage recovery of iron added to samples of serum. The columns represent the number of figures varying from 2.5 per cent above to 2.5 per cent below the designated percentage except for the end sections. At the left the column represents three cases below 72.5 per cent recovery, one as low as 55 per cent.

Using this factor to calculate the recovery of iron in experiments carried out on a number of patients, results were obtained as shown in Fig. 2 in the form of frequency distribution. It was soon apparent that there were two groups, (1) hypochromic anemia and (2) all others. In the normal and miscellaneous cases it is evident that the figures are grouped about the median in a more or less normal manner with a slight skewness which indicates merely that one is more likely to find less than to find more than the added amount of iron. In the case of hypochromic anemia, however, the nonrecovery of iron is more than a technical error. It appears likely that there is something in the serum that interferes with the recovery of iron. An attempt to study this effect led to the following observations, which will be given in brief:

1. Iron added to the supernatant after separation from the precipitate was recovered completely in all cases. From this it was concluded that in these cases the iron was removed along with the protein precipitate (co-precipitation).

2. Doubling the acid in the initial digestion increased the yield of iron where iron had been added but made no difference in the final result in the case of serum without addition. From this it was concluded that the interfering factor acted only on the added iron and did not interfere with the determination of the serum iron.

3. When ferrous rather than ferric iron was added, the yield approximated 100 per cent. This indicated the formation of some sort of ferric iron complex which was carried down partially with the proteins and which could be dissociated by increasing the acidity of the initial digestion.

4. The nature of this complex and whether or not it has any physiologic implications have not been studied.

DETERMINATION OF IRON IN WHOLE BLOOD[1, 5, 12, 13, 14, 15, 16, 17]

A. *Procedure.*—Place 0.1 ml. of whole blood in a 100 ml. Kjeldahl flask. Add 1 ml. concentrated H_2SO_4 and a glass bead and heat the mixture over a microburner until white fumes appear. Then reduce the flame and add a drop of concentrated HNO_3. As soon as the reaction has subsided, add another drop of HNO_3. A third drop will usually be sufficient to complete the ashing. After the H_2SO_4 residue has cooled, transfer it to a 25 ml. volumetric flask and make up to the mark. Transfer 1 ml. of this to glass-stoppered cylinder and determine the iron as in the procedure described previously, beginning with step 5.

One precaution must be observed. After the color has been extracted with ethyl acetate, it may, on standing, acquire an orange tone. The colorimetry must therefore be completed within two minutes after adding the ethyl acetate. This is ample time for the procedure, but no more than one sample should be prepared at one time.

Blank: Dilute 1 ml. of concentrated H_2SO_4 and 3 drops of nitric acid to 25 ml. and determine the iron on 1 ml. of this, as described previously.

B. *Standardization.*—A standard iron preparation was prepared to contain 50 μg per milliliter. The following amounts were used: 1, 0.8, 0.6, 0.4, and 0.2 ml., respectively; each sample was subjected to the procedure of ashing, dilution, and colorimetry, as described previously. The results are contained in Table V. As may be seen, the relationship between color density and concentration of iron follows Beer's law over the range studied, which includes the great majority of the values likely to be encountered. The constant representing the conversion factor (7.6) is lower than in the case of serum, indicating a greater color density. No attempt was made to recover added iron since it was possible to test the method by comparing the total iron determined in this way with the hemoglobin iron determined on a sample from the same blood specimen, in the case of normal bloods.

TABLE V. DETERMINATION OF THE CONVERSION FACTOR FOR USE IN THE CASE OF ASHED WHOLE BLOOD

IRON					
IN ORIGINAL SAMPLE (γ)	AS DETERMINED (γ)	G	L_G	L_C	F_C
50	2.0	52	.284	.259	7.7
40	1.6	58[2]	.232	.207	7.7
30	1.2	65	.187	.162	7.4
20	0.8	74	.131	.106	7.5
10	0.4	83[2]	.077	.052	7.6
Blank		94[2]	.025		

C. *Calculation.*—The L value for the iron in the sample of whole blood is determined as follows: (L_G − L value of Blank) × 25. This × 7.6 (conversion factor) = micrograms in the sample of whole blood used or, if 0.1 ml. is used, milligrams per 100 ml. of whole blood.

The hemoglobin iron in milligrams per 100 ml. is calculated from the value for hemoglobin in grams per 100 ml. by multiplying it by 3.4, since hemoglobin contains 0.34 per cent of iron or 3.4 mg. per gram. The hemoglobin should be determined on the same specimen. In the present work it was done by the method described for the Evelyn colorimeter. One-tenth milliliter of whole blood is diluted with distilled water to 50 ml. One drop of ammonia is added and the solution is read in the colorimeter using filter No. 540 with the galvanometer set at 100 for distilled water. The value for L_G is divided by 0.0258 to obtain the hemoglobin in grams per 100 ml., and this is multipled by 3.4 to obtain hemoglobin iron in milligrams per 100 ml. of blood. Normally there should be little, if any, difference between the determined value for the total iron of the whole blood and the iron as calculated from the hemoglobin.[12-17] But in some types of case the total iron may be appreciably increased above the hemoglobin iron. This is what is meant by the nonhemoglobin iron. Actually in the normal, the total

iron may be slightly above the hemoglobin iron—not more than 2 or 3 per cent. This amount is well above that found in the serum, so that one may safely assume that it is present in the red cells in some form not combined with hemoglobin.

TEST OF THE METHODS

It was not found to be practicable to test the method for serum iron against any of the so-called "standard" or accepted methods. Moreover, it may be doubted whether this is good

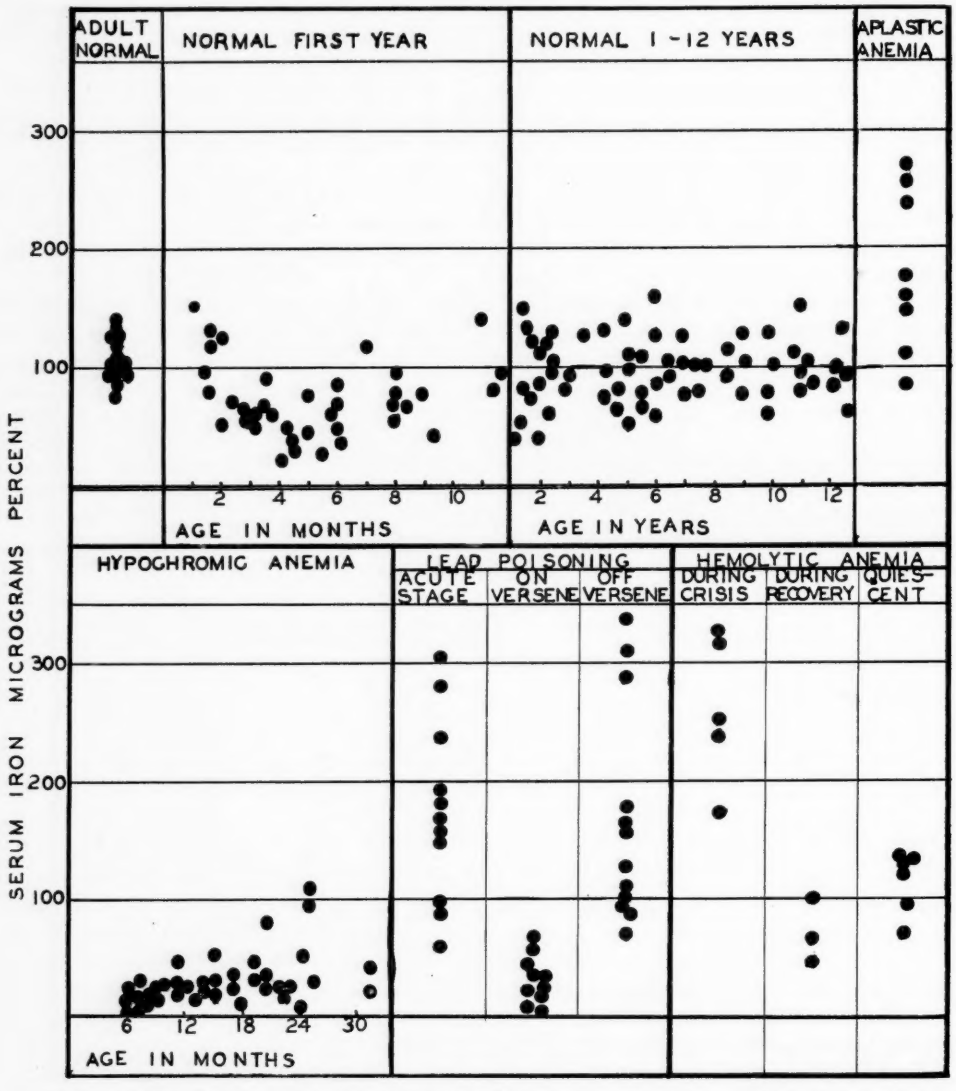

Fig. 3.—Values for serum iron found in infancy and childhood. These figures furnish a test of the method, since they correspond in general to those reported by others: not only the normal average and range, but the variations under certain conditions, e.g., the high values of aplastic anemia, the low values of hypochromic anemia, etc. The effect of Versene on the high levels of lead poisoning is particularly striking.

procedure in testing any method, even though it may lead to a more ready "acceptance" of the new procedure. In lieu of this, determinations made on a number of children and adults under both normal and pathologic conditions are recorded in the form of a chart divided into panels (Fig. 3).

To test the method for the whole blood iron the determined values were compared with those calculated for the iron of the hemoglobin. These comparisons are recorded in Fig. 4, in the form of a frequency distribution. Figures from cases of hypochromic anemia being treated with iron have been included to illustrate the presence of iron in the red cells beyond that to be accounted for by the hemoglobin and the error of the method.

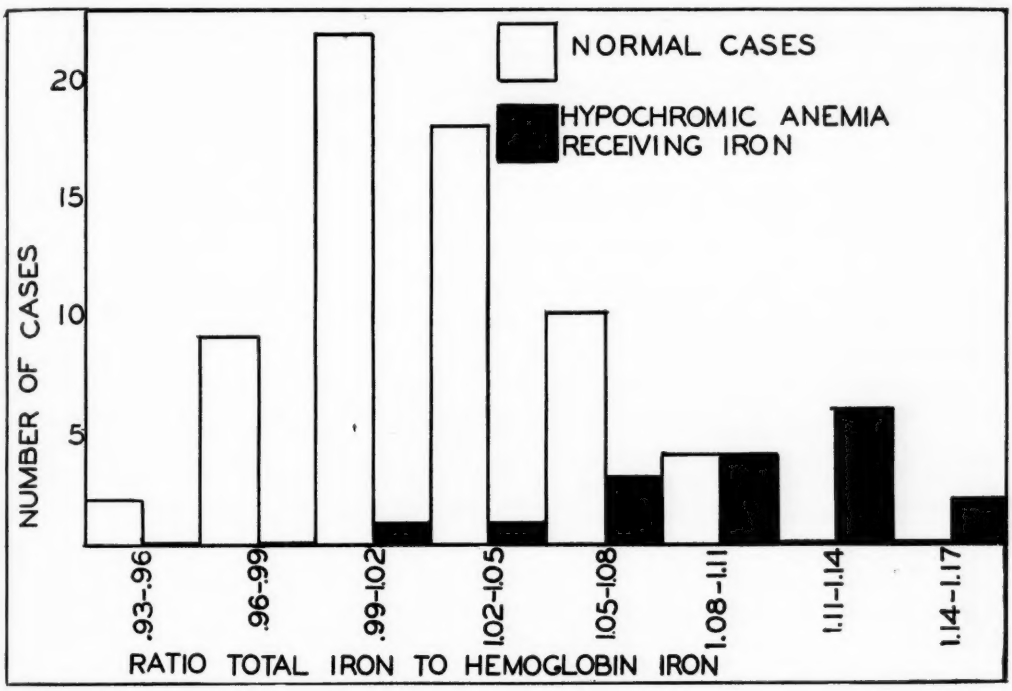

Fig. 4.—Relation between total iron (determined) and hemoglobin iron (calculated) in the whole blood. It is evident that ordinarily there is good correspondence between the two values. This constitutes a practical test of the adequacy of the method. The cases of hypochromic anemia being treated with iron are included to illustrate what is meant by non-hemoglobin iron, since it is clear that in some of these cases there is considerably more iron present than can be accounted for by the hemoglobin. Since the difference may come to 3 or 4 mg. per cent, or even more, the amount is far above any possible level of serum iron and, therefore, can only be present in the red cells themselves. In order to avoid including cases with the probability of larger error, cases with hemoglobin levels below 6 gm. per 100 ml. were excluded. This would correspond to 20 mg. per cent of whole blood or 0.8 μg as determined.

SUMMARY

A method is described for determining iron in as little as 0.5 ml. serum, using thiocyanate for the color, and ethyl acetate extract for the colorimetry, adapted for the "micro" attachment of the Evelyn colorimeter.

A procedure is also described for iron in whole blood which does not differ in its essentials from other procedures in use for the same purpose over the last twenty years, but which has been adapted in its final steps to the "micro" Evelyn colorimeter.

REFERENCES

1. Jenkins, C. E., and Thomson, M. L.: The Distribution of Iron in the Blood, Brit. J. Exper. Path. **18**: 175, 1937.
2. Heilmeyer, L., and Plötner, K.: Das Serumeisen und die Eisenmangelkrankheit, Jena, 1937.

3. Moore, C. V., Minnich, V., and Welch, J.: Studies in Iron Transportation and Metabolism, J. Clin. Invest. **18**: 543, 1939.
4. Kitzes, G., Elvehjem, C. A., and Schuette, H. A.: The Determination of Blood Plasma Iron, J. Biol. Chem. **155**: 653, 1944.
5. Kennedy, R. P.: The Quantitative Determination of Iron in Tissues, J. Biol. Chem. **74**: 385, 1927.
6. Stugart, R.: Determination of Iron in Milk and Other Biological Materials, Ind. and Eng. Chem. Anal Ed. 3, 390, 1931.
7. Moore, C. V., Arrowsmith, W. R., Quilligan, J. J. and Read, J. T.: Studies in Iron Transportation and Metabolism. I. Chemical Methods, J. Clin. Invest. **16**: 613, 1937.
8. Vannotti, A., and Delachaux, A.: Iron Metabolism, Translated by E. Pulay, New York, 1949, Grune and Stratton.
9. Laurell, C. B.: Studies on the Transportation and Metabolism of Iron, Acta physiol. scandinav. **14**: Supp. 46, 1947.
10. Barkan, G., and Walker, B. S.: Determination of Serum Iron With o-Phenanthrolin, J. Biol. Chem. **135**: 37, 1940.
11. Vahlquist, B.: Das Serumeisen, Acta Paedist. 28 supp. 5, 1940.
12. Wong, S. V.: Colorimetric Determination of Iron and Hemoglobin in Blood, J. Biol. Chem. **77**: 409, 1928.
13. Fowweather, F. S.: The Determination of Iron in Blood, Tissues and Urine, Biochem. J. **20**: 93, 1926.
14. Josephs, H. W.: The Iron of the Blood, Bull. Johns Hopkins Hosp. **56**: 50, 1935.
15. Josephs, H. W., and Winocur, P.: The Occurrence of Non-Hemoglobin Iron in Whole Blood, Bull. Johns Hopkins Hosp. **61**: 75, 1937.
16. Lambrechts, A., and Thimus, A.: Le fer total du sang et le fer non-hémoglobinque des globules rouges. Bull. Soc. Chim. Biol. **29**: 1025, 1947.
17. Pagliardi, E.: Dimostrazione della presenza in particolari condizioni fisiopathologiche di ferro non-hemoglobinico nei globuli rossi de sangue umano. Haematologica **34**: 449, 1950.
18. Kato, K., and Iob, V.: Influence of Cobalt on Iron Transportation, Am. J. Clin. Path. **10**: 751, 1938.

METABOLIC AND HISTOLOGIC STUDIES IN PATIENTS WITH AND WITHOUT LIVER DISEASE RECEIVING CHLORAMPHENICOL AND OXYTETRACYCLINE

WILLIAM W. FALOON, M.D.
SYRACUSE, N. Y.
WITH THE TECHNICAL ASSISTANCE OF JOAN W. NOLL, B.S., AND KATHLEEN COLLINS, B.S.

CERTAIN untoward metabolic and nutritional effects of chloramphenicol and oxytetracycline in human beings have been reported, but relatively little quantitative data concerning these effects are available. Vitamin deficiency signs and symptoms of steatorrhea have been observed with these and other antibiotic agents, and certain untoward hepatic effects, possibly nutritional in origin, have been attributed to oxytetracycline.[1-5] Although reported to be of benefit in infectious hepatitis[6] and in hepatic coma,[7] oxytetracycline has nevertheless been indicted in the production of fatty infiltration of the liver when administered orally to human beings,[8, 9] and when given parenterally to animals.[10] Chlortetracycline, similar to oxytetracycline in both chemical structure and antibacterial spectrum, has likewise been found to produce fatty infiltration of the liver in man when given orally, while hyperbilirubinemia has been observed during its parenteral administration.[8, 9, 11, 12, 13] Increased urine nitrogen excretion, accompanied by increased riboflavin excretion, has also been observed during the administration of chlortetracycline without definite correlation with changes in fecal flora.[12, 14] Such urine nitrogen loss has not been corrected by the administration of methionine supplements.[12] Gabuzda and associates[15] have reported that oxytetracycline is also capable of increasing urinary nitrogen excretion.

This study has been undertaken in an attempt to compare the metabolic effects of oxytetracycline and chloramphenicol, and to determine the relationship of hepatic sequelae to such metabolic alterations. Our results with the use of oxytetracycline are in agreement with those of Gabuzda and associates, but we have extended the studies to include riboflavin excretion, fat absorption, hepatic histology, and hepatic function.

MATERIALS AND METHODS

Chloramphenicol* was administered orally in doses of 2 Gm. daily to two patients with fatty cirrhosis of the liver, and to two patients who were undergoing slow resolution of pneumococcal pneumonia previously treated with penicillin. The diets offered to these patients are shown in Table I. The patients varied in their ability to eat constant diets, but the daily intakes were sufficiently uniform to allow valid determination of nitrogen

From the Department of Medicine, State University of New York, Upstate Medical Center, and University Hospital of the Good Shepherd, Syracuse, N. Y.
These studies were aided in part by a grant from Parke, Davis & Company.
Received for publication, Feb. 12, 1954.
*Chloromycetin obtained through the courtesy of Dr. E. C. Vonderheide, Parke, Davis & Company.

balance. Food variety was provided and dietary intake was controlled by rotating three different daily diets equal in protein, fat, and calorie content.

Oxytetracycline* was given to five patients, three of whom had cirrhosis of the liver, one with ascites. A fourth patient was suffering from asthma and varicose veins, and the fifth was undergoing slow resolution of pneumococcal pneumonia previously treated with penicillin. One patient received a diet similar to those offered the patients receiving chloramphenicol, while the others received diets similar in calorie and protein content but low in sodium (Table I.) Two of these received supplementary sodium during part of the study. In one patient, J. B., the dietary intake varied widely during the control period, but sufficient data are available to suggest that a significant change in his nitrogen metabolism occurred during the administration of the antibiotic.

TABLE I

PATIENT	INITIAL WEIGHT (POUNDS)	DIAGNOSIS	DIET	
			PROTEIN (GM.)	CALORIES
Chloramphenicol				
C. B.	134	Resolving pneumonia	58	2,100
W. L.	156	Cirrhosis	80	2,600
J. T.	123	Resolving pneumonia	77	2,500
H. D.	203	Cirrhosis	100	2,500
Terramycin				
J. B.	163	Asthma, varicose veins	63	2,000
R. R.	114	Cirrhosis, ascites	100	2,700*
F. H.	145	Resolving pneumonia	80	2,100*
I. H.	169	Cirrhosis	94	2,400*
H. J.	176	Cirrhosis	100	2,500*

*Low sodium diet.

Duplicates of the diets offered to both groups of patients were analyzed for nitrogen and fat content, and appropriate calculations from standard tables were made for those patients in whom constant intakes were not obtained.† Riboflavin content of the diets was calculated from standard tables.[16] Analyses of each antibiotic for riboflavin revealed no demonstrable riboflavin in either.

Metabolic studies were conducted using four- to seven-day control periods, four- to eight-day periods of antibiotic administration, and subsequent control periods of from two to six days. Twenty-four-hour urine collections were preserved with toluol, and, when necessary, aliquots were acidified, stored in brown bottles, and frozen for subsequent riboflavin analyses. The stools were preserved with measured amounts of benzoic acid, and collection periods were marked by the ingestion of carmine red. Nitrogen in food, urine, and feces was determined by the Macro-Kjeldahl method, and fat in food and feces was determined by the method of Fowweather.[17]

In three patients receiving chloramphenicol, and in two receiving oxytetracycline, liver biopsies were performed before, and at the end of, the course of the antibiotic. Liver biopsies were obtained with a Vim-Silverman needle, using the intercostal approach, and repeated specimens in each patient were obtained through the same intercostal space. Liver function tests included thymol turbidity, using a modification of MacLagan's method,[18] cephalin flocculation,[19] serum bilirubin,[20] and bromsulfalein retention (forty-five minutes after a dose of 5 mg. per kilogram of weight). Urine riboflavin was determined by the fluorometric method of Connor and Straub[21] in three patients receiving chloramphenicol, and in two patients receiving oxytetracycline.

In all patients given chloramphenicol, and in two subjects given oxytetracycline, complete blood counts, hematocrit, and direct eosinophil counts were carried out three times

*Terramycin obtained through the courtesy of Dr. H. A. Strade, Chas. Pfizer and Co., Inc.

†Nitrogen analyses of the antibiotics and capsules revealed an average added nitrogen intake from this source of 250 mg. daily. These values are not included in the calculations.

weekly. In three of the patients receiving chloramphenicol, bone marrow aspiration was performed before and after the administration of this antibiotic. These hematologic studies will be the subject of a separate report.

RESULTS

Chloramphenicol.—

Nitrogen balance: None of the patients receiving chloramphenicol revealed changes in nitrogen metabolism which could be attributed to the effect of the antibiotic (Table II, Fig. 1). In one patient, nitrogen balance changed from negative to slightly positive during the antibiotic period, but this was accompanied by the ingestion of more calories and nitrogen during this period. Likewise in this patient, the postantibiotic period revealed increasingly positive nitrogen balance, suggesting that the chloramphenicol was not responsible for the apparent anabolism. There was no difference in the nitrogen balance response of patients with liver disease, as compared to the patients convalescing from infections.

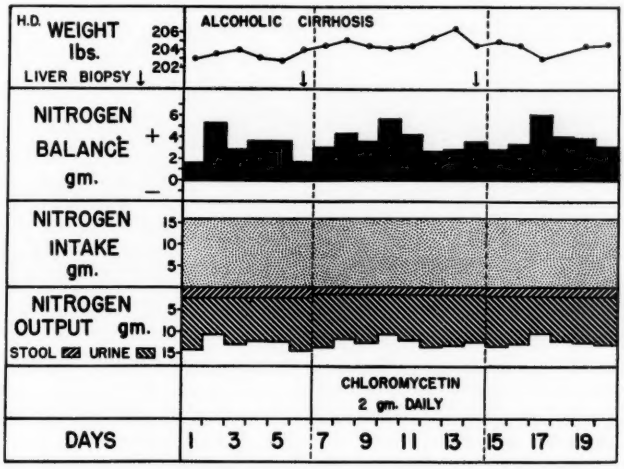

Fig. 1.—Nitrogen balance during chloramphenicol administration.

Liver biopsies and liver function: In three patients receiving chloramphenicol, two of whom had fatty infiltration and fibrosis, the histologic appearance of the liver as revealed by biopsy either remained the same or showed slight improvement after the administration of this antibiotic. Liver function tests similarly showed no change or improvement during this period.

Fat absorption: The excretion of fat in the feces underwent no significant changes when chloramphenicol was given (Table III). None of these patients demonstrated a significant degree of diarrhea, although one had a moderate increase in the number of the stools during and after reception of this agent.

Urine riboflavin excretion: Riboflavin excretion was not significantly altered by the administration of chloramphenicol in the three patients studied (Table

TABLE II. NITROGEN BALANCE DATA IN PATIENTS RECEIVING CHLORAMPHENICOL

	DAY	CALORIES	NITROGEN (GM.) INTAKE	URINE	STOOL	BALANCE	DAY	CALORIES	NITROGEN (GM.) INTAKE	URINE	STOOL	BALANCE
		C. B. Resolving Pneumonia						*J. T. Resolving Pneumonia*				
Control	1	1,587	5.5	7.2	1.3	−3.0	1	2,538	12.5	8.8	2.6	+1.1
	2	1,816	7.3	6.1	1.3	− .1	2	2,391	12.3	8.2	2.6	+1.5
	3	2,085	8.0	5.8	1.3	+ .9	3	2,577	12.4	8.8	2.6	+1.0
	4	2,118	9.3	9.8	1.3	−1.8	4	2,538	12.5	6.8	2.6	+3.1
	5	2,081	9.2	5.4	1.3	+2.5						
	6	2,266	9.4	9.8	1.3	−1.7						
Chloramphenicol 2 Gm. daily	7	2,150	9.4	12.5	1.2*	−4.3	5	2,463	12.3	8.3	1.7	+2.3
	8	2,064	9.2	7.0	1.2	+1.0	6	2,577	12.5	8.6	1.7	+2.2
	9	2,246	9.4	9.5	1.2	−1.3	7	2,538	12.5	9.2	1.7	+1.6
	10	2,167	9.4	7.9	1.2	+ .3	8	2,463	12.3	8.0	1.7	+2.6
	11	2,072	9.2	7.5	1.2	+ .5	9	2,637	12.5	9.1	1.7	+1.7
	12	2,069	8.8	4.7	1.2	+2.9	10	2,538	12.5	7.7	1.7	+3.1
Control	13	2,063	8.9	5.9	1.2*	+1.8	11	2,463	12.3	8.2	2.3	+1.8
	14	2,068	9.2	7.3	1.2	+ .7	12	2,637	12.5	6.9	2.3	+3.2
	15	2,248	9.4	6.3	1.2	+1.9	13	2,538	12.5	7.5	3.3	+2.7
	16	2,167	9.4	7.0	1.2	+1.2	14	2,463	12.3	9.2	2.3	+0.8
	17	2,060	9.1	6.2	1.2	+1.7						
	18	2,266	9.4	5.9	1.2	+2.3						

TABLE II.—CONT'D

	DAY	CALORIES	NITROGEN (GM.) INTAKE	URINE	STOOL	BALANCE	DAY	CALORIES	NITROGEN (GM.) INTAKE	URINE	STOOL	BALANCE
		H. D. Cirrhosis							*W. L. Cirrhosis*			
Control	1	2,513	15.7	11.9	2.2	+1.6	1	2,167	9.4	14.2	.9	−5.7
	2	2,504	15.9	8.4	2.2	+5.3	2	2,230	10.4	11.4	.9	−1.9
	3	2,499	15.9	10.8	2.2	+2.9	3	2,795	13.9	10.1	.9	+2.9
	4	2,453	15.7	9.9	2.2	+3.6	4	2,167	9.4	11.9	.9	−3.4
	5	2,504	15.9	10.1	2.2	+3.6	5	2,230	10.4	10.7	.9	−1.2
	6	2,499	15.9	12.0	2.2	+1.7	6	2,381	11.7	10.4	.9	+ .4
Chloramphenicol 2 Gm. daily	7	2,513	15.7	11.6	1.1	+3.0	7	2,442	11.7	9.3	1.2	+1.2
	8	2,504	16.1	10.7	1.1	+4.3	8	2,230	10.4	9.2	1.2	.0
	9	2,498	16.1	11.4	1.1	+3.6	9	2,541	11.7	8.9	1.2	+1.6
	10	2,512	15.9	9.3	1.1	+5.5	10	2,442	11.7	†	1.2	—
	11	2,504	16.1	10.9	1.1	+4.1	11	2,230	10.4	10.4	1.2	−1.2
	12	2,498	16.1	12.4	1.1	+2.6	12	2,541	11.7	9.0	1.2	+1.5
	13	2,393	16.0	12.0	1.1	+2.9	13	2,442	11.7	8.8	1.2	+1.7
	14	2,421	15.8	11.2	1.1	+3.5						
Control	15	2,498	16.1	11.4	1.9	+2.9	14	2,298	10.4	7.1	1.4	+1.9
	16	2,427	16.3	11.1	1.9	+3.4	15	2,678	12.8	8.6	1.4	+2.8
	17	2,504	16.1	8.3	1.9	+5.9	16	2,580	12.8	8.7	1.4	+2.7
	18	2,498	16.1	10.2	1.9	+4.1	17	2,505	12.7	7.4	1.4	+3.9
	19	2,412	16.5	10.7	1.9	+3.9	18	2,679	12.8	11.1	1.4	+ .3
	20	2,504	16.1	11.1	1.9	+3.1						

*Second and third period stools were pooled together.
†Urine collection incomplete.

IV, Fig. 2). Although in patient C.B., the urinary excretion of riboflavin fell markedly during the antibiotic period, this effect persisted during the second control period and was associated with the occurrence of positive nitrogen balance. Urinary excretion was more constant in the other two patients, but a similar relationship between nitrogen and riboflavin was observed. In no patient was there an increase in the excretion of riboflavin when chloramphenicol was given.

TABLE III. FAT INTAKE AND FECAL FAT DURING ANTIBIOTIC ADMINISTRATION (GRAMS PER DAY)

PATIENT	PERIOD	DAYS	INTAKE	FECES	LOSS (%)
C. B.	Control	6	57.7	1.9	3.3
	Chloramphenicol	6	65.7	1.8*	2.7
	Control	6	65.7	1.8*	2.7
J. T.	Control	4	86.8	6.5	7.5
	Chloramphenicol	6	86.8	3.7	4.3
	Control	4	86.8	4.5	5.2
W. L.	Control	6	77.8	2.7	3.5
	Chloramphenicol	7	79.1	2.9	3.7
	Control	5	86.1	3.5	4.1
H. D.	Control	6	93.5	10.5	11.2
	Chloramphenicol	8	90.8	3.0	3.3
	Control	6	90.0	5.3	5.9
J. B.	Control	7	41.7	2.3	5.5
	Terramycin	4	59.9	1.7*	2.9
	Control	2	59.9	1.7*	2.9
F. H.	Control	5	76.6	7.3	9.5
	Terramycin	6	76.6	7.4	9.7
	Terramycin	6	76.6	6.3	8.2
	Terramycin	6	76.8	4.0	5.2
	Control	4	76.8	7.0	9.1
R. R.	Control	7	93	7.8	8.3
	Terramycin	6	93	5.9	6.4
	Control	5	92.5	7.7	8.4
	Aureomycin	6	88.6	10.1	11.4
	Control	6	88.8	7.2	8.1
I. H.	Control	6	89.5	3.5	3.9
	Terramycin	6	89.5	3.8	4.2
	Control	6	89.5	3.6	4.3
H. J.	Control	6	98.1	1.1	1.2
	Terramycin	6	100.6	3.5	3.5
	Control	4	100.6	4.0	4.0

*Stool collections were pooled for the two consecutive periods indicated.

Oxytetracycline.—

Nitrogen balance: The administration of oxytetracycline resulted in increased urine nitrogen excretion in four of the five patients (Table V, Figs. 3 and 4). One patient, F. H., (Fig. 5) showed no significant change in urine nitrogen or nitrogen balance from the preantibiotic control period, but after the antibiotic was stopped, urine nitrogen excretion fell, and nitrogen balance became slightly positive. This patient received oxytetracycline during successive periods of (a) low sodium intake, (b) 3 Gm. of added sodium chloride daily, and (c) ad libitum sodium chloride. The results indicate that there was no significant alteration of nitrogen balance during administration of the antibiotic as a result of the addition of sodium to an otherwise constant diet.

In patient J. B., whose study was marred by variation in his dietary intake and by loss of urine specimens, the urine nitrogen appeared to increase markedly

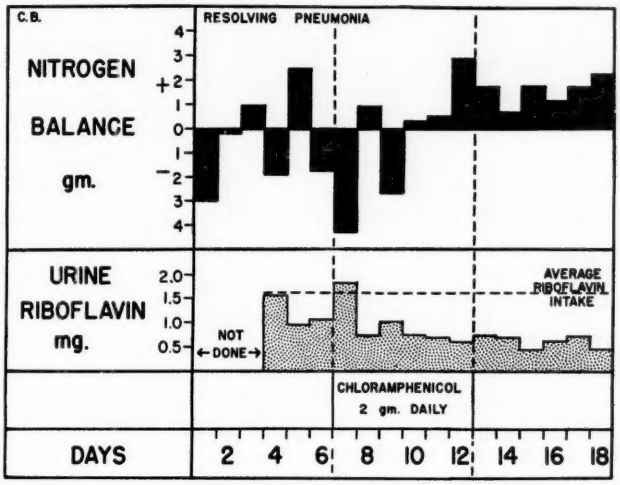

Fig. 2.—Nitrogen balance and urine riboflavin excretion during chloramphenicol. Urine riboflavin decreased as positive nitrogen balance occurred, without relation to the administration of the antibiotic.

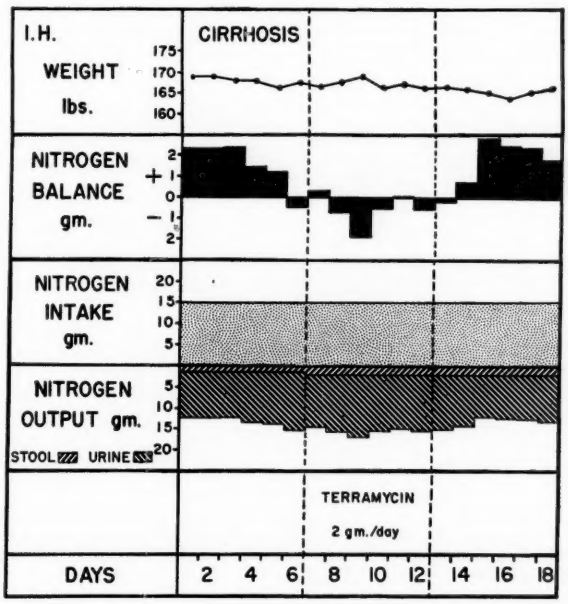

Fig. 3.—Negative nitrogen balance during oxytetracycline administration.

during the antibiotic period. This occurred despite somewhat greater caloric intake during this period than had been accomplished previously. Negative nitrogen balance ensued in this patient and reversal of this effect was not clearly apparent during the two-day period after withdrawal of the antibiotic.

In patient R. R., who had cirrhosis with ascites, the administration of oxytetracycline produced an insignificant change in urine nitrogen excretion as compared to the last five days of the initial control period, but in the second

TABLE IV. URINE RIBOFLAVIN EXCRETION IN PATIENTS RECEIVING CHLORAMPHENICOL OR OXYTETRACYCLINE (MG. PER DAY)

	CHLORAMPHENICOL—2 GM. DAILY						OXYTETRACYCLINE—2 GM. DAILY			
	C. B.		H. D.		W. L.		I. H.		H. J.	
DAY	AVERAGE DAILY INTAKE	URINE	AVERAGE DAILY INTAKE	URINE	AVERAGE DAILY INTAKE	URINE	AVERAGE DAILY INTAKE	URINE	AVERAGE DAILY INTAKE	URINE
1		----	2.26	2.667	1.92	2.297	4.37	2.086‡	4.40	2.726
2		----		2.242		1.659		2.086‡		2.979
3		----		1.961		1.153		2.479		3.091
4	1.61	1.580		1.312		2.855		2.919		3.084
5		.958		1.717		2.863		3.139		2.602
6		1.081		1.950		2.596		5.107		2.862
7	1.59	1.861*	2.27	1.622*	2.18	2.284*	4.37	3.121†	4.42	3.493†
8		.776*		1.592*		2.024*		4.747†		3.769†
9		1.021*		1.519*		2.223*		5.255†		4.939†
10		.724*		1.283*		.544*		4.204†		2.098†
11		.694*		.719*		1.918*		3.074†		2.613†
12		.615*		1.383*		1.223*		5.108†		4.578†
13	1.60	.716		1.317*		1.606*	4.36	2.758	4.43	3.3836
14		.710		1.128*	2.54	1.776		3.848		2.0569
15		.441	2.28	1.276		1.872		2.960		2.069
16		.611		1.397		2.011		2.998		1.467
17		.709		1.233		1.278		2.562		----
18		.408		1.547		2.088		2.736		----
19		----		1.446		----		----		----
20		----		1.474		----		----		----

Average daily intake is the average for the period indicated.
*Days when receiving chloramphenicol.
†Days when receiving oxytetracycline.
‡Urine pooled for these two days.

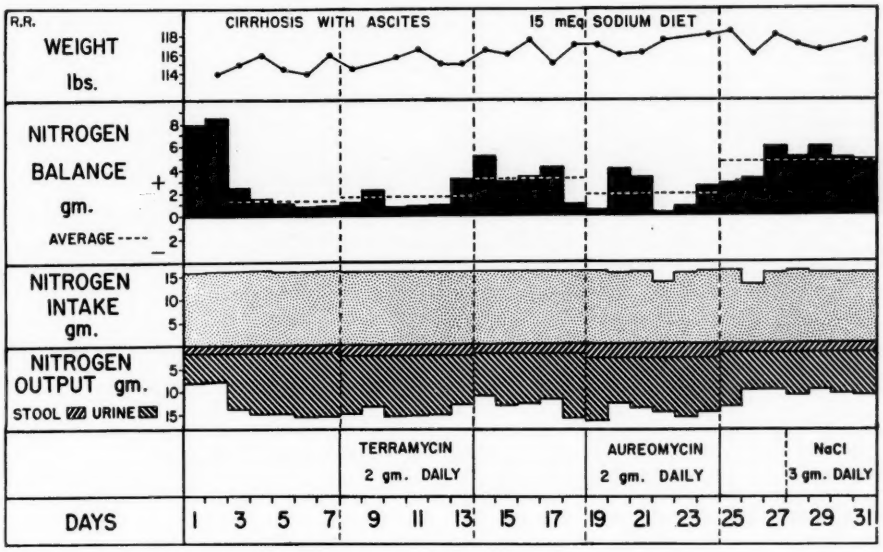

Fig. 4.—Nitrogen balance during oxytetracycline and chlortetracycline administration. Less positive balance occurred during these periods than during control intervals except for initial control period. (Dotted line indicates average nitrogen balance for each period. Average balance during initial control period calculated for the last five days only).

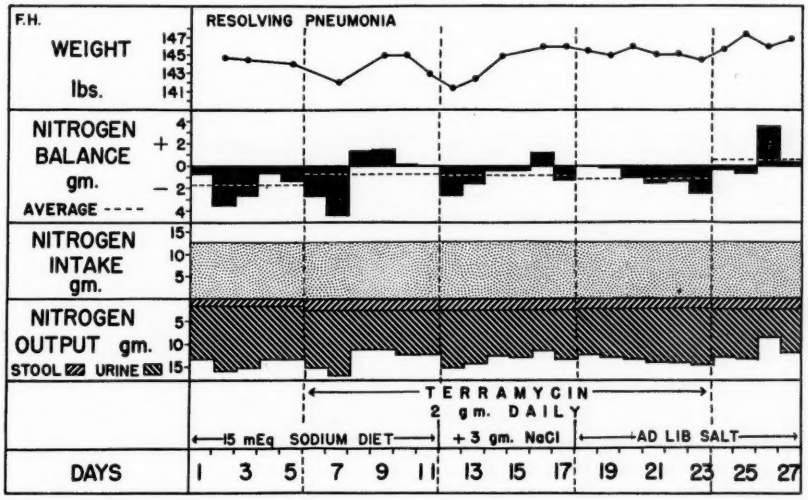

Fig. 5.—Nitrogen balance during oxytetracycline administration. No significant alteration noted, although slightly positive balance occurred after antibiotic was stopped. Increased sodium intake was without effect on nitrogen metabolism. (Dotted line indicates average nitrogen balance for each metabolic period.)

control period, increased retention of nitrogen occurred (Fig. 4). After the second control period, this patient was given chlortetracycline,* 2 Gm. daily, in an attempt to compare the effect of the two antibiotics. With this antibiotic, the nitrogen balance was altered to a degree quite similar to that observed with

*Aureomycin obtained through the courtesy of Dr. Stanton M. Hardy, Lederle Laboratories Division, American Cyanamid Company.

TABLE V. NITROGEN BALANCE DATA IN PATIENTS RECEIVING OXYTETRACYCLINE

			NITROGEN						NITROGEN						NITROGEN			
	DAY	CALORIES	INTAKE	URINE	STOOL‡	BALANCE-	DAY	CALORIES	INTAKE	URINE	STOOL	BALANCE-	DAY	CALORIES	INTAKE	URINE	STOOL	BALANCE-
	J. B., Asthma						*I. H., Cirrhosis*						*H. J., Cirrhosis*					
Control	1	2,069	10.5	7.3	1.4	+1.8	1	2,380	15.1	11.5	1.3	+2.3	1	2,420	15.8	7.7	0.3	+7.8
	2	1,482	6.3	7.9	1.4	−3.0	2	2,348	15.1	11.5	1.3	+2.3	2	2,670	15.8	10.1	0.3	+5.4
	3	1,272	4.2	5.6	1.4	−2.8	3	2,389	15.1	11.4	1.3	+2.4	3	2,717	15.8	9.1	0.3	+6.4
	4	1,509	9.1	*	1.4	---	4	2,420	15.1	12.4	1.3	+1.4	4	2,672	15.8	8.9	0.3	+6.6
	5	1,521	7.0	*	1.4	---	5	2,348	15.1	12.6	1.3	+1.2	5	2,582	15.8	9.3	0.3	+6.2
	6	2,012	10.1	6.8	1.4	+1.9	6	2,374	15.1	14.3	1.3	−0.5	6	2,704	15.8	7.9	0.3	+7.6
	7	1,762	10.1	7.3†	1.4	+1.4†												
Terramycin 2 Gm. daily	8	1,943	10.1	8.1	1.4	+ .6	7	2,420	15.1	12.8	2.0	+ .3	7	2,696	16.0	11.5	1.5	+3.0
	9	1,969	10.1	10.7	1.4	−2.0	8	2,348	15.1	13.8	2.0	− .7	8	2,779	16.0	11.0	1.5	+3.5
	10	1,852	9.8	14.2	1.4	−5.8	9	2,405	15.1	15.0	2.0	−1.9	9	2,704	16.0	10.5	1.5	+4.0
	11	1,803	10.1	12.5	1.4	−3.8	10	2,420	15.1	13.6	2.0	− .5	10	2,696	16.0	10.1	1.5	+4.4
							11	2,420	15.1	13.1	2.0	.0	11	2,779	16.0	11.4	1.5	+3.1
							12	2,414	15.1	13.6	2.0	− .5	12	2,704	16.0	12.8	1.5	+1.7
Control	12	1,892	9.7	12.9	1.4	−4.6	13	2,420	15.1	13.2	2.1	− .2	13	2,696	16.0	13.7	2.0	+0.3
	13	2,109	10.1	10.9	1.4	−2.2	14	2,420	15.1	12.2	2.1	+ .8	14	2,779	16.0	10.5	2.0	+3.5
							15	2,405	15.1	10.2	2.1	+2.8	15	2,704	16.0	11.8	2.0	+2.2
							16	2,420	15.1	10.5	2.1	+2.5	16	2,687	16.0	7.7	2.0	+5.3
							17	2,420	15.1	10.6	2.1	+2.4						
							18	2,405	15.1	11.5	2.1	+1.5						

TABLE V.—CONT'D

	DAY	CALORIES	NITROGEN INTAKE	NITROGEN URINE	NITROGEN STOOL	NITROGEN BALANCE		DAY	CALORIES	NITROGEN INTAKE	NITROGEN URINE	NITROGEN STOOL	NITROGEN BALANCE
	F. H., Resolving Pneumonia								*R. R., Cirrhosis*				
Control	1	2,076	12.9	11.9	1.7	− .7	Control	1	2,570	15.8	6.4	1.6	+7.8
	2	2,064	12.5	14.4	1.7	−3.6		2	2,639	16.0	5.9	1.6	+8.5
	3	2,095	12.7	13.7	1.7	−2.7		3	2,655	16.1	12.1	1.6	+2.4
	4	2,086	13.0	11.9	1.7	− .6		4	2,711	16.4	13.3	1.6	+1.5
	5	2,013	12.5	13.1	1.7	−2.3		5	2,602	16.0	13.3	1.6	+1.1
								6	2,688	16.0	13.6	1.6	+ .8
								7	2,687	16.1	13.6	1.6	+ .9
Oxytetracycline	6	2,095	12.7	12.8	2.7	−2.8	Oxytetracycline	8	2,687	16.0	12.8	2.0	+1.2
	7	2,065	13.0	14.6	2.7	−4.3		9	2,688	16.0	11.0	2.0	+3.0
	8	2,065	12.5	8.6	2.7	+1.2		10	2,687	16.1	13.3	2.0	+ .8
	9	2,095	12.7	8.6	2.7	+1.4		11	2,685	16.0	13.1	2.0	+ .9
	10	2,086	13.0	10.2	2.7	+ .1		12	2,688	16.0	13.0	2.0	+1.0
	11	2,065	12.5	10.0	2.7	− .2		13	2,687	16.1	10.8	2.0	+3.3
Oxytetracycline	12	2,095	12.7	12.7	2.6	−2.6	Control	14	2,685	16.0	9.0	1.8	+5.2
	13	2,086	13.0	11.9	2.6	−1.5		15	2,688	16.0	11.2	1.8	+3.0
	14	2,065	12.5	10.3	2.6	− .4		16	2,687	16.1	10.9	1.8	+3.4
	15	2,095	12.7	10.5	2.6	− .4		17	2,685	16.0	10.0	1.8	+4.2
	16	2,086	13.0	9.2	2.6	+1.2		18	2,688	16.0	14.1	1.8	+ .1
	17	2,065	12.5	11.1	2.6	−1.2							
Oxytetracycline	18	2,095	12.7	10.7	2.1	− .1	Chlortetracycline	19	2,686	16.1	12.6	2.8	+ .7
	19	2,086	13.0	11.0	2.1	− .1		20	2,514	15.7	8.8	2.8	+4.1
	20	2,065	12.5	11.5	2.1	−1.1		21	2,688	16.0	10.0	2.8	+3.2
	21	2,095	12.7	12.1	2.1	−1.5		22	2,353	13.8	10.8	2.8	+ .2
	22	1,999	12.9	12.2	2.1	−1.4		23	2,562	15.7	12.1	2.8	+ .8
	23	1,978	12.5	12.8	2.1	−2.4		24	2,688	16.0	10.7	2.8	+2.5
Control	24	2,095	12.7	10.9	2.2	− .4	Control	25	2,687	16.1	11.5	1.8	+2.8
	25	2,075	13.0	11.4	2.2	− .6		26	2,259	13.1	8.1	1.8	+3.2
	26	2.049	12.5	6.7	2.2	+3.6		27	2,639	15.8	8.2	1.8	+5.8
	27	2,095	12.7	10.0	2.2	+ .5		28	2,650	16.1	9.2	1.8	+5.1
								29	2,569	15.7	8.1	1.8	+5.8
								30	2,616	15.7	8.9	1.8	+5.0
								31	2,574	15.8	9.3	1.8	+4.7

*Urine lost.
†Incomplete urine collection.
‡Stool for all periods pooled together.

oxytetracycline. Also, in the final control period, after chlortetracycline was stopped, markedly positive balance ensued in a fashion similar to the post-oxytetracycline period. The addition of sodium chloride to the diet for four days in the latter period was without apparent effect on nitrogen metabolism.

In patients I. H. (Fig. 3) and H. J., both suffering from cirrhosis, urine nitrogen rose and fecal nitrogen increased slightly during the administration of oxytetracycline. The combination of these two factors yielded less positive nitrogen balance in one patient and negative nitrogen balance in the other during the antibiotic period.

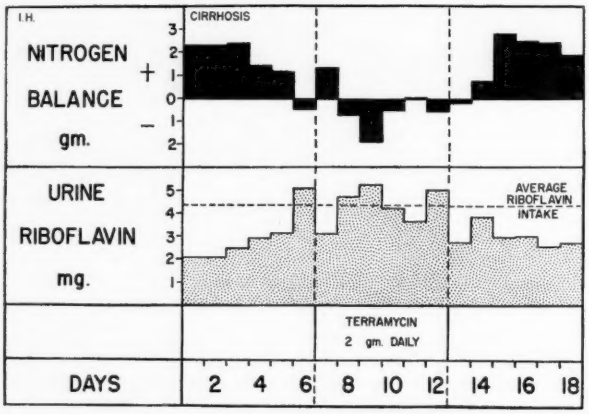

Fig. 6.—Nitrogen balance and urine riboflavin during oxytetracycline. Urine riboflavin increase coincided with increased nitrogen loss when antibiotic was administered.

Fecal fat: In none of the five patients receiving oxytetracycline (Table III), was there an increase in fecal fat excretion, although in two, I. H. and H. J., an increase in number and in total volume of the stools occurred. The extension of the period of antibiotic administration to as long as eighteen days, as in patient F. H., also did not produce steatorrhea.

Liver biopsy and liver function tests: Liver biopsies were carried out before and after oxytetracycline administration in patients I. H. and H. J. In the latter, there was a slight, probably insignificant, increase in the amount of fatty infiltration, and, in the other patient, no alteration in liver histology was observed. Liver function tests showed no change or slight improvement in these patients during the course of antibiotic administration.

Riboflavin excretion: In both patients in whom urinary riboflavin was determined, significant increase in the excretion of this vitamin was observed (Table IV, Fig. 6), during the administration of oxytetracycline. This increase corresponded to the increase in nitrogen excretion and was reversed when the antibiotic was stopped. Although the urinary loss of riboflavin was more consistent and more striking in I. H. than in H. J., the average excretion during oxytetracycline administration in the latter patient was 3.58 mg. daily, as compared to 2.89 mg. and 2.24 mg. during the preantibiotic and postantibiotic periods, respectively.

DISCUSSION

It is apparent from the data obtained that nitrogen balance is unchanged by the administration of chloramphenicol, but that an increase in nitrogen excretion of varying degree occurs in most patients receiving oxytetracycline. The latter result confirms the results obtained by Gabuzda and his colleagues,[15] and suggests that oxytetracycline and chlortetracycline are comparable in metabolic effects, as well as in antibacterial effects and chemical structures. In the study in which these compounds were compared in the same patient, the results indicate similar quantitative effects upon nitrogen metabolism. In this experiment, the failure of oxytetracycline to increase the excretion of nitrogen as compared to the preantibiotic control period may perhaps be explained by the observation of Gabuzda and Davidson[22] that certain patients with hepatic disease attain nitrogen anabolism more slowly than do patients without liver disease given a comparable adequate diet. Thus, the oxytetracycline may have inhibited the occurrence of more positive nitrogen balance in the second week of this dietary regimen. This possibility is strengthened by the fact that markedly positive balance did occur in the postoxytetracycline period, and even more positive balance subsequently was observed in the third control, or postchlortetracycline period. These findings suggest that the development of progressively greater anabolism in this patient was interrupted successively by oxytetracycline and chlortetracycline.

The mechanism of the effect produced by oxytetracycline cannot yet be defined, but it appears unlikely that changes in the gastrointestinal bacterial flora are responsible. Using chlortetracycline, Gabuzda and associates found no consistent quantitative or qualitative change in stool flora which correlated with the increase in urine nitrogen excretion.[14] In addition, chloramphenicol has been reported by some, but not all, investigators[23] to alter the gastrointestinal flora. Fecal flora studies were not carried out in this study, but if alterations in the flora occurred with chloramphenicol, no concomitant nitrogen balance changes were observed in contrast to the results with the other two antibiotics which have been studied in this manner. On the other hand, chloramphenicol may not have been given for sufficiently long periods to alter the flora severely enough to affect nitrogen balance. In this regard, it has been reported by McGovern and associates that fungus growth in the gastrointestinal tract occurs maximally in children after chloramphenicol has been administered for fourteen days.[24] Despite the possibility that these periods of study were too brief to allow marked changes in the flora, it would appear more probable that some intrinsic chemical property of oxytetracycline and chlortetracycline, rather than the fecal flora changes, renders these compounds, in contrast to chloramphenicol, capable of producing the catabolic effect. In regard to this possibility, however, Gabuzda and colleagues using "alkaline rearranged," antibiotically inactive chlortetracycline have been unable to obtain increased nitrogen excretion as previously observed when the active form of this agent was used.[15] Increased calorie loss caused by steatorrhea and resultant increased nitrogen catabolism induced by these agents can be eliminated on the basis of

our fecal lipid studies. In regard to nitrogen metabolism, chloramphenicol is less deleterious to protein metabolism than the other agents and, therefore, would appear to be preferable in patients in whom nitrogen anabolism is desirable.

The finding of increased urine nitrogen excretion during the administration of oxytetracycline is of interest in regard to the findings of Bateman and associates,[25] who have described marked rises in plasma nonprotein nitrogen in patients receiving this antibiotic intravenously in large doses. It is possible that the nitrogen catabolism observed by us may be partly responsible for such azotemia, although diminished renal function also appears to have been produced in their patients.

Since most of the patients receiving oxytetracycline were fed low-sodium diets, in contrast to those receiving chloramphenicol, the possible relationship of this factor to the catabolic effect of oxytetracycline was briefly explored in patients R. R. and F. H. The results obtained did not suggest that the intake of sodium significantly affected the metabolic alterations produced by this agent.

Although clinical signs of riboflavin deficiency have been reported during therapy with chloramphenicol and oxytetracycline, urinary riboflavin excretion in our patients was related to protein metabolism, with increasing loss being associated with nitrogen catabolism in the course of oxytetracycline administration. This relationship has previously been observed by Pollack and associates[26] in other catabolic states. Although it is possible that interference with the proper utilization of this vitamin occurred as a result of the administration of oxytetracycline, we have no evidence indicating that riboflavin deficiency was produced without nitrogen loss. Using chlortetracycline which produces a similar increase in urine riboflavin excretion, Gabuzda and colleagues have been unable to prevent increased nitrogen loss by the use of riboflavin supplements.[15] Such evidence suggests that either riboflavin and nitrogen loss are inseparable in such circumstances, or that the metabolic blockade produced by chlortetracycline is not competitively reversible by the use of vitamin supplements. Regardless of the mechanism of these nutritional alterations, it is readily apparent that chloramphenicol differs from oxytetracycline and chlortetracycline in that neither riboflavin nor nitrogen loss is induced by its administration.

The absence of change in hepatic histology in the patients receiving chloramphenicol is consistent with the results obtained by Lepper and associates in animals.[10] Although oxytetracycline did not produce significant fatty change in the two patients in this study, the period of administration may have been too brief to produce such alteration. Similar brief intervals of chlortetracycline administration in previous studies also failed to produce fatty infiltration in patients with liver disease, although fatty infiltration was observed with prolonged periods of administration.[12] From these results, it appears that increased nitrogen excretion is either unrelated to fatty infiltration of the liver, or precedes it, in patients receiving these antibacterial agents.

Absorption of fat was unaltered despite the occurrence of increased fecal volumes in one patient receiving chloramphenicol, and in two patients receiv-

ing oxytetracycline. This is also in accord with observations in patients receiving chlortetracycline,[12] and suggests that even when fecal volume is increased, steatorrhea does not develop. These results, however, may not apply to all patients receiving these agents.

SUMMARY AND CONCLUSIONS

Nitrogen balance, riboflavin excretion, fat absorption, and liver histology and function were studied in patients with and without liver disease receiving either chloramphenicol or oxytetracycline.

No alteration in nitrogen balance was observed during the administration of chloramphenicol to four patients, but in four of five patients receiving oxytetracycline, urine nitrogen excretion was significantly increased. Riboflavin excretion was related to nitrogen balance; no change occurred with the use of chloramphenicol, but significant increases occurred during the nitrogen catabolism observed when oxytetracycline was given.

Fat absorption was not affected by either antibacterial agent, despite the occurrence of increased fecal volumes in some patients. Similarly, liver histology and function tests either improved or were unchanged by these agents during the brief, four- to eight-day periods of administration used in these studies.

It is concluded that oxytetracycline, like chlortetracycline, is capable of producing nitrogen catabolism accompanied by increased riboflavin excretion. No definite correlation of this effect with changes in hepatic histology was observed. Chloramphenicol, however, does not affect nitrogen metabolism, riboflavin excretion, or hepatic function. The effects of the administration of chloramphenicol or oxytetracycline upon nitrogen metabolism are unrelated to the presence or absence of liver disease.

We are indebted to Misses Gloria Rossi and Phyllis Utter and to Mrs. Clarice Shepard for the preparation and administration of the diets used. Our gratitude is also expressed to Dr. John T. Prior, who reviewed the pathologic specimens.

REFERENCES

1. Gewin, H. M., and Friou, G. J.: Manifestations of Vitamin Deficiency During Aureomycin and Chloramphenicol Therapy of Endocarditis Due to Staphylococcus Aureus: Report of Case, Yale J. Biol. & Med. **23**: 332, 1951.
2. Leitner, Z. A.: Vitamin Deficiency and Antibiotics, Brit. M. J. **1**: 491, 1950.
3. Williams, B., Jr.: Oral and Pharyngeal Complications of Chloramphenicol Therapy, Am. Pract. & Digest. Treat. **1**: 897, 1950.
4. Harris, H. J.: Aureomycin and Chloramphenicol in Brucellosis With Special Reference to Side Effects, J. A. M. A. **142**: 161, 1950.
5. Merliss, R. R., and Hoffman, A.: Steatorrhea Following the Use of Antibiotics, New England J. Med. **245**: 328, 1951.
6. Colbert, J. W., Jr., Bungards, L., and Knowlton, M.: Aureomycin, Chloromycetin and Terramycin in Treatment of Acute Viral Hepatitis, Proc. Soc. Exper. Biol. & Med. **79**: 339, 1952.
7. Ducci, H., and Katz, R.: Cortisone, ACTH and Antibiotics in Fulminant Hepatitis, Gastroenterology **21**: 357, 1952.
8. Yesner, R., and Kunkel, P.: Preliminary Observations on Effect of Aureomycin, Terramycin, Tibione, Combined Tibione and Streptomycin and Chloromycetin on Morphology of Liver in Man, Yale J. Biol. & Med. **23**: 299, 1951.
9. Sborov, V. M., and Sutherland, D. A.: Fatty Liver Following Aureomycin and Terramycin Therapy in Chronic Hepatic Disease, Gastroenterology **18**: 598, 1951.

10. Lepper, M. H., Zimmerman, H. J., Carroll, G., Caldwell, E. R., Spies, H. W., Dowling, H. F., and Wolfe, C. K.: Effect of Large Doses of Aureomycin, Terramycin and Chloramphenicol on Livers of Mice and Dogs, Arch. Int. Med. **88**: 284, 1951.
11. Lepper, M. H., Wolfe, C. K., Zimmerman, H. J., Caldwell, E. R., Spies, H. W., and Dowling, H. F.: Effect of Large Doses of Aureomycin on Human Liver, Arch. Int. Med. **88**: 271, 1951.
12. Faloon, W. W., Noll, J. W., and Prior, J. T.: Nitrogen Metabolism and Liver Histology During Aureomycin Administration in Patients With Hepatic Disease, J. LAB. & CLIN. MED. **41**: 596, 1953.
13. Rutenburg, A. M., and Pinkes, S.: The Hepatotoxicity of Intravenous Aureomycin, New England J. Med. **247**: 797, 1952.
14. Gabuzda, G. J., Jackson, G. G., and Grigsby, M. E.: Effect of Aureomycin Upon Nitrogen Balance and Urinary Riboflavin Excretion in Undernourished Men, J. Clin. Invest. **31**: 631, 1952.
15. Gabuzda, G. J., Gocke, T. M., and Love, B. D., Jr.: Metabolic Effects of Antibiotics Administered to Undernourished Men, J. Clin. Invest. **32**: 570, 1953.
16. Jolliffe, N., Tisdall, F. F., and Cannon, P. R.: Clinical Nutrition, New York, 1950, Paul B. Hoeber, Inc.
17. Fowweather, F. S.: The Determination of the Amount of Composition of the Fat of Faeces. I. Investigation of a "Wet" Method and Comparison With the "Dry" Method, Brit. J. Exper. Path. **7**: 7, 1926.
18. Maclagan, N. F.: Thymol Turbidity Test: A New Indicator of Liver Dysfunction, Nature, London **154**: 670, 1944.
19. Hanger, F. M.: Serological Differentiation of Obstructive From Hepatogenous Jaundice by Flocculation of Cephalin-Cholesterol Emulsions, J. Clin. Invest. **18**: 261, 1939.
20. Ducci, H., and Watson, C. J.: The Quantitative Determination of the Serum Bilirubin With Special Reference to the Prompt Reacting and the Chloroform-Soluble Types, J. LAB. & CLIN. MED. **31**: 910, 1945.
21. Connor, R. T., and Straub, G. J.: Combined Determination of Riboflavin and Thiamin in Food Products, Ind. and Eng. Chem. Anal. Ed. **13**: 385, 1941.
22. Gabuzda, G. J., and Davidson, C. S.: Protein Metabolism in Patients With Cirrhosis of the Liver, Ann. New York Acad. Sc. (In press.)
23. Kirsner, J. B., Levin, E., and Palmer, W. L.: Use of Antibiotics in Gastrointestinal Diseases, Arch. Int. Med. **90**: 677, 1952.
24. McGovern, J. J., Parrott, R. H., Emmons, C. W., Ross, S., Burke, F. G., and Rice, E. C.: The Effect of Aureomycin and Chloramphenicol on the Fungal and Bacterial Flora of Children, New England J. Med. **248**: 397, 1953.
25. Bateman, J. C., Barberio, J. R., Grice, P., Klopp, C. T., and Pierpoint, H.: Fatal Complications of Intensive Antibiotics Therapy in Patients With Neoplastic Disease, Arch. Int. Med. **90**: 763, 1952.
26. Pollack, H., and Bookman, J. J.: Riboflavin Excretion as a Function of Protein Metabolism in the Normal Catabolic and Diabetic Human Being, J. LAB. & CLIN. MED. **38**: 561, 1951.

FECAL FAT ON AN ESSENTIALLY FAT-FREE DIET

GEORGE T. LEWIS, PH.D., AND HELEN C. PARTIN, M.S.
MIAMI, FLA.

UNDER usual circumstances of diet and activity in normal adults, the feces contain about one-fourth of their dry weight as lipid material. In view of the insolubility of fats in the aqueous medium of the body, and the possible formation of insoluble soaps with salts of calcium and magnesium, the most obvious explanation would seem to be incomplete absorption of dietary fat. Variations in amount and type of fat eaten, however, are not the major influence governing the amount in the stool. In addition, the composition of the fecal lipid fairly closely resembles that found in the blood. If a dietary source is eliminated, other possibilities are: bacteria present in the stool, desquamated intestinal epithelium, and excretion by the mucosa of the intestinal wall. Bacteria and cellular elements can be rather definitely eliminated,[1] leaving excretion as the most probable origin of the fecal fat. This point of view has received wide, but not entirely universal, acceptance.[2]

The majority of the experimental foundation for this statement has come from work on dogs. While the results are presumably applicable to human beings, one can be certain only when the experiments have been repeated on human subjects. A recent compilation,[3] however, contains no figures on the fecal lipid excretion of human beings on a low fat diet.

The critical experiment would seem to be the maintenance of adults on a fat-free diet for fairly extended periods, with concomitant collection of feces, and analysis of the specimens for lipids. We have recently had the opportunity to carry out such an experiment and the results form the basis of this communication.

MATERIALS AND METHODS

In this laboratory a feeding regimen designed for continuous enteral administration to patients with feeding problems has been developed. The composition is given in Table I. The solution contains no added lipid and only 10 mg. per liter of ether-soluble material. How much of this is fatty acid or glyceride, we do not know, but the fatty acid intake cannot be more than 25 mg. per day and is probably considerably below this figure.

Stool specimens were collected in twenty-four-hour periods and preserved in a cold room at approximately +2° C. until analysis. Prior to the application of the analytic procedures, the twenty-four-hour specimen was homogenized in a Waring Blendor. When necessary sufficient water was added to make a fluid mass, and aliquots were measured volumetrically. The aliquot for nitrogen determination was placed in a Kjeldahl flask and the nitrogen content determined by the usual Kjeldahl technique. Another aliquot was transferred to a container with a rather large surface and evaporated to dryness on the water bath. The dry powder was placed in a filter paper thimble and extracted exhaust-

From the Medical Research Foundation of Dade County.
Received for publication Feb. 22, 1954.

ively with ether in a Soxhlet extractor. The ether was removed by evaporation and the extract weighed. This value is designated total fat. Addition to the extract of ether gave a solution from which aliquots were removed for the determination of fatty acids by the method of Stewart and Hendry,[4] of cholesterol (free and total) by the technique of Schoenheimer and Sperry,[5] and of lipid phosphorus according to Fiske and SubbaRow.[6]

TABLE I. FORMULA FOR FEEDING SOLUTION

	PER LITER
Protein	65 Gm.
Hydrolyzed lactalbumin	
Carbohydrate	160 Gm.
Mineral	
Sodium	1300 mg.
Potassium	700 mg.
Calcium	1200 mg.
Magnesium	170 mg.
Phosphorus	300 mg.
Iron	1.5 mg.

RESULTS

The results of the individual analyses are given in Table II, and these are summarized in Table III. Reference to the data will show that the total fecal fat has no relation to the nitrogen excretion, which might be an argument against the origin of the fat in bacteria or desquamated cells. The amount of fat in the stool varies widely, which is perhaps surprising in view of the constancy of the diet. The average excretion is about 2 Gm., however. This has been noted previously,[7] but the basis for the statement is not given. As indicated, no attempt was made to prevent hydrolysis during the interval between collection and analysis. Therefore, we have no means of estimating the amount of triglyceride originally present. Additional fatty acid may have been excreted in the form of soaps or volatile acids. No attempt was made to determine these fractions.

TABLE II. FECAL LIPID

DATE	N_2 GM.	TOTAL FAT GM.	FATTY ACID (%)	CHOLESTEROL TOTAL (%)	CHOLESTEROL FREE (%)	LIPID P (%)
			Patient 1			
5/26	10.7	1.63	38	9		
6/4	2.8	0.97	23	16	13	0.10
6/9	2.2	0.53	34	34	32	
6/11	1.1	0.29	29	26		
6/15	2.2	0.60	34	26		
6/20	0.93	0.37	25	24		
6/21	2.1	0.27	20	22		
6/24	5.1	0.13	38	21		
7/4	3.9	1.2	22	25	22	0.08
7/6	3.8	2.4	30	13		
7/6	4.4	2.25	22	27	22	
7/23	6.2	5.4	28	15		0.06
			Patient 2			
2/12	4.8	3.59	55	25		0.27
2/16	10.0	2.35	49			
2/18	7.9	1.74	45			
			Patient 3			
2/18	3.5	2.66	50	12		0.22

TABLE III. FECAL LIPID: SUMMARY

	N_2 (GM.)	TOTAL FAT (GM.)	FATTY ACID (%)	CHOLESTEROL TOTAL (%)	LIPID P AS LECITHIN (%)
Av. No. 1	3.8	1.3	29	20	2.0
Av. No. 2	7.6	2.6	50	25	6.8
Av. No. 3	3.5	2.7	50	12	5.5

DISCUSSION

A disadvantage of the experimental conditions is the fact that the subjects were not normally active adults. They were hospital patients living under the usual hospital regime. Patient 1 was an adult man living in the hospital following a hemi-mandibulectomy for a radiation necrosis. He was awaiting plastic surgery and had no other recognizable pathology. Patient 2 was a young woman recovering from ulcerative colitis and Patient 3 was a middle-aged man with multiple peptic ulcers.

There is at least a theoretical possibility that ether-soluble compounds in the bile might contribute to the total fat fraction. Qualitative tests for bile acids were negative for these preparations, however.

SUMMARY

The entire ether-soluble material and certain of the fractions which make up this total have been determined on twenty-four-hour stool specimens from three adult human subjects. In one case, such determinations were carried out at intervals during a period of two months. The diet, although nutritionally adequate, contained a daily maximum of 25 mg. of fatty acid. Under these conditions, the daily excretion of lipid approximated 2 Gm.

These data are consistent with the belief that fecal lipid represents excretion by the intestinal mucosa.

REFERENCES

1. Sperry, Warren M.: Lipid Excretion. VIII. The Lipid Content of the Intestinal Mucosa, J. Biol. Chem. **96**: 759, 1932.
   Sperry, Warren M., and Angevine, Robert W.: IX. The Secretion of Lipids Into the Intestine, J. Biol. Chem. **96**: 769, 1932.
2. Wollaeger, Eric E., Comfort, Mandred W., and Osterberg, Arnold E.: Total Solids, Fat and Nitrogen in the Feces: III. A Study of Normal Persons Taking a Test Diet Containing a Moderate Amount of Fat; Comparison With Results Obtained With Normal Persons Taking a Test Diet Containing a Large Amount of Fat, Gastroenterology **9**: 272, 1947.
3. Cook, R. P.: Comparative Aspects of Lipid Absorption and Excretion, Lipid Metabolism, Biochemical Society Symposia No. 9, Cambridge, 1952, Cambridge University Press, p. 23.
4. Stewart, C. P., and Hendry, E. B.: The Determination of Total Fatty Acids in Blood, Biochem. J. **29**: 1683, 1935.
5. Schoenheimer, R., and Sperry, W. M.: A Micromethod for the Determination of Free and Combined Cholesterol, J. Biol. Chem. **106**: 745, 1934.
   Sobel, A. E., and Mayer, A. M.: Improvements in the Schoenheimer-Sperry Method for the Determination of Free Cholesterol, J. Biol. Chem. **157**: 255, 1945.
6. Fiske, C. H., and SubbaRow, Y.: The Colorimetric Determination of Phosphorus, J. Biol. Chem. **66**: 375, 1925.
7. White, A.: Diseases of Metabolism. Lipid Metabolism: Digestion of Lipids, Philadelphia, 1952, W. B. Saunders Co., p. 200.

ACQUIRED ANTITHROMBOPLASTINOGENEMIA (HEMOPHILIA-LIKE DISEASE): WITH SPECIAL REFERENCE TO ITS DIAGNOSIS

ARMAND J. QUICK, PH.D., M.D., EINAR R. DANIELS, M.D., AND CLARA V. HUSSEY, M.S.
MILWAUKEE, WIS.

LOZNER, Jolliffe, and Taylor[1] discovered, in 1940, a new anticoagulant distinct from heparin which caused a hemorrhagic diathesis simulating hemophilia. They noted that the addition of their patient's plasma to normal blood significantly prolonged the clotting time, a finding which is not observed when hemophilic plasma is mixed with normal blood. Their observation has become the basis for diagnosing the presence of the anticoagulant and for assaying its content. For the latter procedure, varying amounts of either the blood[2] or plasma[3] are added to a fixed volume of normal blood and the clotting time of the mixture then determined. Quick and Stefanini[4] observed that blood containing the anticoagulant will, when added to normal blood, not only increase the clotting time, but depress the prothrombin consumption time. The latter finding has been confirmed in additional cases by Singer and associates,[5] Pons and de Torregrosa,[6] Frick,[7] and Nilsson and Wenckert.[8] In this paper, two cases are reported in which dependence on the clotting time test alone could easily have resulted in failure to establish the presence of the anticoagulant, whereas, the prothrombin consumption test and the thromboplastinogen activity time not only made the detection of the inhibitory agent unequivocal, but furnished a simple means for assaying its concentration, thereby serving as means for following the course of the disease quantitatively.

REPORT OF CASES

CASE 1.—C. K., a white police officer, aged 48 years, had no history of a bleeding tendency prior to June, 1952, when he noted an abnormally large bruise after hitting his shin on a lawn mower. This was the first sign of a bleeding tendency. Nothing in his previous history furnishes significant information concerning the possible origin of the condition. Two years earlier he had an outer ear infection which was treated with a sulfonamide, but it had to be stopped because of the patient's sensitivity to the drug; it is not possible to say whether this had any relation to his present illness. On August 18 he had abdominal cramps; later, he had pain in left upper quadrant and noticed blood in his urine. A cystoscopic examination done three days later showed that the bleeding came from the pelvis of the left kidney. He was admitted to the Milwaukee Hospital the following day. The pain had shifted to the mid-epigastrium region and localized in the right lower quadrant. The intensity and localization suggested acute appendicitis.

From the Department of Biochemistry, Marquette University School of Medicine, Milwaukee.

This investigation was supported by a grant (H-1612) from the Grants and Training Branch of the National Heart Institute, United States Public Health Service.

Received for publication Feb. 26, 1954.

Upon operation, a retroperitoneal hematoma in the right kidney was found. The appendix was removed. Severe bleeding from the incision, which required the transfusion of 15 pints of blood during the next three days, began eight hours postoperatively. The incisional bleeding was partly controlled by pressure, and gross hematuria ended on the third postoperative day. The initial laboratory findings following one transfusion were essentially normal, including a clotting time of eight minutes. Later, a prolonged clotting time of over one hour directed attention to a coagulation disturbance. The first studies to determine the nature of the hemorrhagic condition were made Sept. 22, 1952, with the following results:

		Normal
Clotting time (Lee-White)	105 min.	5-8 min.
Bleeding time (Duke)	2 min.	1½-3 min.
Tourniquet test	Negative	
Prothrombin time	12½ sec.	12 sec.
Total prothrombin time	8 sec.	8-9 sec.
Platelet count	163,000	250,000
Clot retraction	Normal	
P.C.T.*	8.0 sec.	15-45 sec.
T.A.T.†	8.0 sec.	15-45 sec.
Thrombin time	8 sec.	8 sec.

*Prothrombin consumption time.
†Thromboplastinogen activity time.

All the procedures were carried out exactly as previously described.[9] For the estimation of the thrombin time, 0.1 ml. of thrombin of varying dilution was added to 0.2 ml. of oxalated plasma. The value for only one concentration of thrombin is recorded, but it was found that even at high dilutions of thrombin, there was no difference between the clotting time of the patient's plasma and the normal plasma.

Since the patient had no history of hemorrhage until his present history, the diagnosis of hemophilia was unlikely, although the laboratory findings suggested a hemophilia-like condition. The presence of a circulating anticoagulant was clearly suggested by the following findings:

Patient's blood	(ml.)	2.0	1.0	0.8	0.5	0.3	0.1	
Normal blood	(ml.)		1.0	1.2	1.5	1.7	1.9	2.0
Clotting time	(min.)	90	14	11	6	5	3½	6
P.C.T.	(sec.)	8	8	8	8½	8½	9½	20
T.A.T.	(sec.)	8	8½	8	9	9½	24	40

For these studies, blood was collected with silicone-coated needles and syringes, and transferred to test tubes similarly coated. To avoid the possible error of tissue contamination, the first blood drawn was discarded and a second syringe used to obtain the blood for the clotting time studies. Mixing of the bloods was done with silicone-coated serologic 1 ml. pipettes calibrated in tenths of a milliliter. The prothrombin consumption time was done on the serum obtained after the clotting time had been determined. The clotted blood was incubated fifteen minutes at 37° C., centrifuged at 2,000 r.p.m. for one minute, then returned to the water bath, and the prothrombin time of the serum determined after thirty and sixty minutes.

The thromboplastinogen activity time test consists in mixing 2 ml. of the blood with 0.05 ml. of rabbit brain thromboplastin heated to 60° C. for twenty minutes. The details of the method have been recently described.[10]

The results obtained by mixing normal and the patient's blood clearly showed the presence of an anticoagulant. It is obvious, however, that the clotting time was not nearly so sensitive a measure of the inhibitor as the prothrombin consumption and the thromboplastinogen activity time tests. When the normal blood and the patient's blood were mixed in equal amounts, the clotting time was fourteen minutes, which is definitely, but not strikingly, longer than normal. In the ratio of one part of patient's blood to three

parts of normal blood, the clotting time was completely normal but the prothrombin consumption test still demonstrated the inhibitory effect of the patient's blood when added in the ratio of one to twenty parts of normal blood. The patient's consistently normal prothrombin time indicated that the anticoagulant is not an antithromboplastin. This was further substantiated by the results obtained by using progressively diluted thromboplastin solutions[11]:

Thromboplastin (mg.*)	200	100	75	50	25	10	5
	Prothrombin time (sec.)						
Patient	12	12	12	16	17½	23	25
Normal I	12	12	12	15	17½	23	24½
Normal II	12	12	12	15	17	22	25

*Quantity of standardized acetone-dehydrated rabbit brain extracted with 5 ml. saline solution.

Although the patient improved clinically, he continued to have subcutaneous and intramuscular hemorrhages usually as the result of slight trauma. He was studied monthly and the results of December 12 are representative:

Patient's blood	(ml.)	1.0	0.75	0.5	0.4	0.3	
Normal blood	(ml.)		0.25	0.5	0.6	0.7	1.0
Clotting time	(min.)	50	5½	5½	6	5	6
P.C.T.	(sec.)	8½	8½	9	10	10	18
Patient's blood	(ml.)	2.0	0.3	0.2	0.1		
Normal blood	(ml.)		1.7	1.8	1.9	2.0	
T.A.T.		10	10	10	12	20	

By means of both the prothrombin consumption time and the thromboplastinogen activity time tests, it was evident that the patient's blood continued to have a high titer of the anticoagulant. The clotting time, on the contrary, failed to detect the inhibitor. Thus, one part of normal blood completely corrected the clotting time of three parts of the patient's blood.

During the period of Jan. 9 to 24, 1953, the patient was given ACTH and, later, cortisone. No clinical improvement was noted and the anticoagulant did not decrease as shown by the results of February 13:

Patient's blood	(ml.)	1.0	0.75	0.5	0.25	
Normal blood	(ml.)		0.25	0.5	0.75	1.0
Clotting time	(min.)	105	10	8	5	6½
P.C.T.	(sec.)	8	8½	9½	8	19

Again, the clotting time gave equivocal results, while the prothrombin consumption time indicated that the titer remained relatively high. No further treatment was attempted but the patient was instructed to apply ice and pressure immediately after any injury. Gradual improvement occurred clinically but the titer of the anticoagulant did not materially decrease as shown by the findings of March 30:

Patient's blood	(ml.)	1.0	0.75	0.5	0.25	
Normal blood	(ml.)		0.25	0.5	0.75	1.0
Clotting time	(min.)	35	8½	7½	6	7½
P.C.T.	(sec.)	8	8	9½	10	28

Improvement continued but the bleeding tendency did not disappear as he still developed severe bruises and intramuscular bleeding from relatively mild contusions and the anticoagulant titer, although diminished, was still sufficiently high to be readily detected as shown by the findings of December 21:

Patient's blood	(ml.)	1.0	0.75	0.5	0.25		2.0	1.0	0.5	0.3	0.2	
Normal blood	(ml.)		1.25	0.5	0.75	1.0		1.0	1.5	1.7	1.8	2.0
Clotting time	(min.)	25	5½	5	4	7						
P.C.T.	(sec.)	9	10	11	13½	40						
T.A.T.	(sec.)						10	15½	31	38	49	

Although a slow progressive improvement has continued for a year, the blood still contains sufficient titer of the anticoagulant to inactivate an equal volume of normal blood, and clinically, the blending tendency continues as shown by intramuscular and subcutaneous hemorrhages occurring after mild trauma.

CASE 2.—J. M., a 9-year-old white boy, was not circumcised because of a hypospadias. At the age of 11 months, it was noted that he bruised easily, and a year later he began having hemarthrosis involving both knees, ankles, and elbows, and numerous intramuscular hemorrhages. For several years, he was comparatively well and had no serious hemorrhages. During the past year, another bleeding cycle began; he had two attacks of hematuria and in June, 1952, he had hemorrhages into his left ankle and knee. After a gradual recovery, he reinjured his knee by falling from a pony. He apparently also injured his back since he began complaining of back pain and after several days had hematuria. It was at this time (Sept. 5, 1952) that the patient was first seen and the following findings obtained:

Clotting time	100 min.
Prothrombin time	14 sec.
Total prothrombin time	8 sec.
Prothrombin consumption time	8½ sec.
Thromboplastinogen activity time	8½ sec.

Patient's blood	(ml.)	2.0	1.5	1.0	
Normal blood	(ml.)		0.5	1.0	2.0
Clotting time	(min.)	100	54	27	6
P.C.T.	(sec.	8½	8½	9	25
T.A.T.	(sec.)	8½	9½	9	28

The findings clearly suggested the presence of an anticoagulant in the blood, but it was not possible from the history to determine the cause with any certainty. Three weeks before these studies were made, he had a virus infection which was treated with Terramycin and penicillin. It should also be stated that the boy has had over thirty transfusions during the past eight years and has received blood intramuscularly. He has furthermore been given injections of a preparation named "neothromboplastin."

The boy has always been considered to be a hemophiliac although the family history is negative. He has one normal brother.

The patient was restudied Oct. 25, 1953, with the following results:

Patient's blood	(ml.)	1.0	0.75	0.5	0.4	0.3	0.1	
Normal blood	(ml.)		0.25	0.5	0.6	0.7	0.9	1.0
Clotting time	(min.)	18	5	5	5	5	5	5
P.C.T.	(sec.)	10	11	11½	13	16	17	23½
Patient's blood	(ml.)	2.0	1.5	1.0	0.6	0.4		
Normal blood	(ml.)		0.5	1.0	1.4	1.6	2.0	
T.A.T.	(sec.)	9	9	9	10	17	24	

The change that occurred in a year is striking, especially the decrease in the clotting time from 100 to 18 minutes. If it were not for the results of the prothrombin consumption time and thromboplastinogen activity tests done on mixtures of the patient's blood with normal blood, the presence of the anticoagulant would have been missed, since one part of normal blood added to three parts of the patient's blood brought the clotting time to five minutes. The persistence of the anticoagulant in remaining in the blood for a long period is well illustrated by this patient.

THROMBOPLASTINOGEN TITRATION TEST

In order to differentiate hemophilia from the hemophilia-like condition caused by the anticoagulant (antithromboplastinogen), a simple procedure was developed. It consists in adding increasing amounts of a standard preparation of thromboplastinogen to a series of test tubes containing a fixed volume of blood, and in determining the prothrombin remaining in the serum after the bloods are clotted under standardized conditions. A small amount of

thromboplastinogen will correct the defective prothrombin consumption in hemophilia, whereas, a much greater quantity is required to increase the prothrombin consumption to normal in blood containing the anticoagulant.

Preparation of Thromboplastinogen.—Ninety milliliters of normal human blood are collected with a silicone-coated syringe and needle, mixed with 10 ml. of 0.1M sodium oxalate in a silicone-coated centrifuge tube, chilled in an ice bath, and centrifuged in an angle centrifuge at 4,000 r.p.m. for twenty minutes. The platelet-poor plasma is deprothrombinized as follows: An 0.012M $Ca_3(PO_4)_2$ suspension equal in volume to that of the plasma is centrifuged to pack the adsorbent. The supernatant water is poured off, and the plasma thoroughly mixed with the $Ca_3(PO_4)_2$ using a silicone-coated stirring rod. The plasma is centrifuged at 12,000 r.p.m. in an angle centrifuge for one hour to remove completely the adsorbent and the remaining platelets. The deprothrombinized and platelet-free plasma is mixed

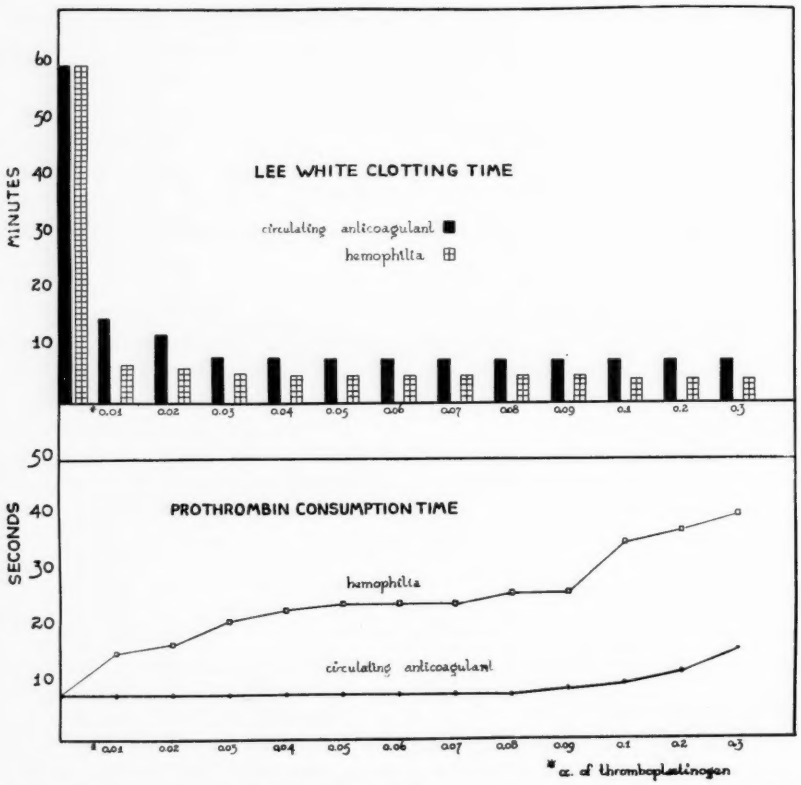

Fig. 1.—The response of the prothrombin consumption to thromboplastinogen in hemophilia and in antithromboplastinogenemia. The thromboplastinogen in the concentrated preparation is about ten times more concentrated than in normal human blood.

with a volume of distilled water, cooled to 2° C. in an ice bath and carbon dioxide is bubbled through the solution. The precipitate, consisting essentially of thromboplastinogen and fibrinogen with some labile factor, but free of prothrombin, platelet factor, and various accessory clotting factors that are adsorbed by $Ca_3(PO_4)_2$, is packed by centrifugation, freed of the supernatant liquid, dissolved in 0.85 per cent saline solution, and brought to a volume one-tenth of that of the plasam from which it was derived.

The Titration.—To a series of test tubes increasing amounts of thromboplastinogen preparation, as indicated in Fig. 1, are transferred and enough saline solution added to bring the volume to 0.3 ml. By means of a silicone-coated serologic pipette, 1 ml. of blood is added to each tube, which is kept in a water bath at 37° C. The clotting time is de-

termined and fifteen minutes later the tube is centrifuged at 2,000 r.p.m. for one minute. The prothrombin of the serum is determined after forty-five minutes of incubation in the water bath.

From the results recorded in Fig. 1, one can plainly see how hemophilia can be differentiated from the condition caused by the inhibitor. Only 0.01 ml. of the thromboplastinogen preparation was required to increase the prothrombin consumption time of 1 volume of hemophilic blood from eight to fifteen seconds. In sharp contrast, 0.09 ml. added to blood containing the inhibitor only increased the prothrombin consumption time from eight to nine seconds, and 0.3 ml. were needed to bring the value to sixteen seconds. Thus, the latter blood required thirty times more thromboplastinogen than hemophilic blood for a comparable correction. The insensitivity of the clotting time to differentiate the two conditions is easily discernible from the chart.

DISCUSSION

The conclusion of Craddock and Lawrence[2] that the circulating anticoagulant inhibits the action of the antihemophilic globulin is in agreement with the views of several other investigators who subsequently studied and reported cases having this inhibitory agent.[5, 7, 12, 13, 14] Both Craddock[2] and Frommeyer[12] and their associates demonstrated specific precipitin titers in the serum of their patients against the antihemophilic globulin, which indicates that the anticoagulant actually inactivates the latter by combining with it. The development of the concept that antihemophilic globulin or thromboplastinogen reacts with a platelet factor to form plasma thromboplastin[15] makes it possible to offer a simple explanation for the action of the anticoagulant. By inactivating thromboplastinogen and, thereby, markedly depressing its concentration in the blood, a condition almost identical with hemophilia is produced. In either disease, little thromboplastin can form and therefore the prothrombin consumption is very low. The fact that the prothrombin time is normal clearly shows that the inhibitor is not an antithromboplastin.

As already pointed out, the data of the present paper supplies further confirmation for the concept that the anticoagulant is an antithromboplastinogen. Its action may be expressed by the equation:

$$\text{Thromboplastinogen} + \text{Inhibitor} \rightleftarrows \text{Thromboplastinogen}\cdot\text{Inhibitor}$$

The greater the amount of the inhibitor, the more is the depression of the concentration of thromboplastinogen. In most of the reported cases, the blood contained such an excess of the anticoagulant that the addition of a small amount to a relatively large volume of normal blood was sufficient to inactivate the thromboplastinogen content of the latter, thereby causing a prolonged clotting time of the mixture.

In the cases presented in the present study, the initial findings also showed a sufficient excess of anticoagulant to prolong the clotting time of normal blood to which the patient's plasma was added. As the patients improved, the anticoagulant apparently diminished sufficiently so that the agent was no longer demonstrable by the clotting time test on normal blood even though still present in relatively high concentration, as indicated by the prothrombin consumption test. The lack of sensitivity of the clotting time test may be explained as follows: The speed of inactivation of thromboplastinogen of nor-

mal blood by the inhibitor is dependent on concentration of the latter. If it is not sufficiently high, a small amount of thromboplastinogen may escape inactivation and react with the platelet component to form a small amount of thromboplastin and a corresponding quantity of thrombin which, though small, is enough to reduce the clotting time to the normal range. This is comparable to thrombocytopenia, in which a normal clotting time continues to be found even when the platelet count is reduced to 3,000 per cubic millimeter or less. Since plasma, which is completely deprived of platelets, is incoagulable, it is obvious that only a small number of platelets is sufficient to reduce the clotting time to normal.

In both patients, the prothrombin consumption and the thromboplastinogen activity tests showed that the anticoagulant continued to remain in the blood in a relatively high titer, while the clotting time test failed completely to detect its presence. The implication of these observations is obvious. Since the clotting time test does not differentiate with certainty true hemophilia from the hemophilia-like disease induced by the anticoagulant, cases of the latter type can easily be mistaken for true hemophilia. It is very probable that the number of cases in which a bleeding condition is caused by the acquired anticoagulant is much higher than is indicated by the few reported cases, since the anticoagulant can easily escape detection if only the clotting time test is used.

The complication of hemophilia by the anticoagulant is especially important for both theoretical and practical reasons. Frommeyer and associates[12] found that five out of a series of twenty-two cases of classical hemophilia contained the anticoagulant, as shown by the clotting time test, and were refractory to treatment by transfusion. It is probable that if the prothrombin consumption test had been used in addition to the clotting time test, the incidence of the anticoagulant occurring in hemophiliacs, especially in those treated by plasma fraction, would have been found even higher. It is likely that the fluctuations in the clotting time and even variations in the clinical course of the disease may perhaps in many cases be traceable to the occurrence of the anticoagulant in the blood. In uncomplicated hemophilia, the clotting time may remain remarkably constant, as recorded in a previous study.[16] The second patient, J. M., who is very likely a true hemophiliac, illustrates how the anticoagulant may distort the basic or primary defect. The clotting time of nearly two hours observed in the first study suggested severe hemophilia, but the drop to eighteen minutes, a year later, makes such a conclusion doubtful. Perhaps, even the periodic changes of his clinical condition may be due to variation in the concentration of the inhibitor.

There is much evidence that the antithromboplastinogen is caused by isoimmunization as Craddock and Lawrence[2] first suggested. It is particularly significant that the inhibitor is rather frequently observed in hemophiliacs who have received numerous transfusions of blood and particularly plasma protein fractions. The development of antithromboplastinogenemia post partum is also in support of the theory of isoimmunization. Particularly interesting is the observation of Frick[7] that the agent even appeared in the blood of a baby

born of a mother who had this bleeding condition following a previous pregnancy. Whether causes other than isoimmunization can bring about the production of the inhibitor is still problematic.

While the causative factor of the antithromboplastinogenemia in Case 1 remains undetermined, it appears fairly certain that transfusions were of no striking benefit and it is possible that they may have actually accentuated the bleeding tendency. It is likely that transfusions and the injection of blood intramuscularly may have induced the condition in the second patient. In neither case has it been determined whether transfusions would cause an increase in the titer of the anticoagulant.

The differentiation between hemophilia, hemophilia complicated by an anticoagulant, and hemophilia-like disease (acquired antithromboplastinogenemia) is of great practical importance since rational therapy is dependent upon it. While fresh blood or plasma will specifically cause a temporary correction of the deficiency in hemophilia (the lack of thromboplastinogen), it may in the other two conditions perhaps stimulate a further production of the inhibitor and accentuate the hemorrhagic state. Craddock and Lawrence[2] cite such an instance. In addition to transfusions, other agents or conditions can bring about antithromboplastinogenemia and intensify the hemorrhagic state after it has been established. Several cases have been reported in which the disease developed post partum.[6, 7, 17, 18, 19] Frick's report is especially noteworthy, as a clinical remission occurred which lasted until a second pregnancy came almost to term, at which time the bleeding tendency again became acute and presumably the anticoagulant titer increased.

The tendency of the disease to improve gradually and even become clinically latent makes the need of a sensitive method for detection and diagnosis particularly urgent. As pointed out, the clotting time test is not sufficiently sensitive to detect mild, or even moderately severe, cases. The prothrombin consumption test carried out on varying mixtures of the patient's with normal blood furnishes a simple means not only to detect the anticoagulant but also to titrate its concentration. The thromboplastinogen titration test is even more promising, since a preparation of fairly uniform potency can be prepared, and the anticoagulant measured in units of thromboplastinogen.

If antithromboplastinogen is detected in the blood, the transfusion of blood or plasma becomes of doubtful value and might even be harmful since it may stimulate the production of more inhibitor as observed by Craddock and Lawrence.[2] Their observation that washed red cells did not cause an increase in the anticoagulant suggests that it is preferable to whole blood or plasma to correct blood loss. The use of plasma seems indicated only if hemorrhagic shock is imminent. Neither cortisone nor ACTH had any beneficial effect on the first patient, which agrees with the observation of other investigators[5, 7, 19, 20] with the exception of Van Creveld and associates,[21] who noted temporary benefit.

SUMMARY

Two cases were a bleeding diathesis in which the causative factor was found to be antithromboplastinogen are described. It was observed that the

prothrombin consumption test carried out on the serum from varying mixtures of the patients' blood with normal blood was a more sensitive test for detecting the inhibitor than was the clotting time. A new test was developed for differentiating hemophilia and antithromboplastinogenemia: It consists of adding increasing quantities of a standardized thromboplastinogen preparation to a fixed volume of blood and determining the prothrombin remaining in the serum. Significantly, greater quantities of thromboplastinogen are required to bring the prothrombin consumption to normal in antithromboplastinogenemia than in hemophilia.

ADDENDUM

On March 26, Patient 1 noted a gradual swelling of his tongue and difficulty in swallowing. This was found to be due to infiltration of blood. Presumably, he had ruptured a small blood vessel in his pharynx from coughing. A few days earlier he had had a relatively mild upper respiratory infection. He was taken immediately to the hospital. The swelling in his throat continued and twenty-four hours later, a tracheotomy had to be performed. In spite of continuous transfusion of blood, the profuse postoperative bleeding continued, blood trickled into his trachea, and gradually filled his bronchial tree, causing death by suffocation. Continuous aspiration of the bronchi by suction was of no avail.

REFERENCES

1. Lozner, E. L., Jolliffe, L. S., and Taylor, F. H. L.: Hemorrhagic Diathesis With Prolonged Coagulation Time Associated With a Circulating Anticoagulant, Am. J. M. Sc. **199:** 318-327, 1940.
2. Craddock, C. G., Jr., and Lawrence, J. S.: Hemophilia: A Report of the Mechanism of the Development and Action of an Anticoagulant in Two Cases, Blood **2:** 505-518, 1947.
3. Conley, C. L., Hartmann, R. C., and Morse, W. I.: Circulating Anticoagulants: A Technique for Their Detection and Clinical Studies, Bull. Johns Hopkins Hosp. **84:** 255, 1949.
4. Quick, A. J., and Stefanini, M.: Activation of Plasma Thromboplastinogen and Evidence of an Inhibitor, Proc. Soc. Exper. Biol. & Med. **67:** 111-112, 1948.
5. Singer, K., Mond, E., Hyman, J., and Levy, R. C.: Circulating Anticoagulants in Hemophilia and in Hemophilia-like Disease, Blood **5:** 1135-1149, 1950.
6. Pons, E. R., rJ., and de Torregrosa, M. V.: Hemorrhagic Diathesis Due to a Circulating Anticoagulant, Blood **7:** 20-30, 1952.
7. Frick, P. G.: Hemophilia-like Disease Following Pregnancy, Blood **8:** 598-608, 1953.
8. Nilsson, I. M., and Wenckert, A.: Hyperglobulinemia as the Cause of Hemophilia-like Disease, Blood **8:** 1067-1077, 1953.
9. Quick, A. J.: The Physiology and Pathology of Hemostasis, Philadelphia, 1951, Lea and Febiger.
10. Quick, A. J., Hussey, C. V., and Stapp, W. F.: The Effect of Heating on the Thromboplastic Activity of Rabbit Brain Extract, a New Test for the Diagnosis of Hemophilia, J. Lab. & Clin. Med. **39:** 142-147, 1952.
11. Quick, A. J., and Hussey, C. V.: Influence of Concentration of Thromboplastin on Prothrombin Time of Human and Dog Plasma, Proc. Soc. Exper. Biol. & Med. **76:** 732-734, 1951.
12. Frommeyer, W. B., Jr., Epstein, R. D., and Taylor, F. H. L.: Refractoriness in Hemophilia to Coagulation-promoting Agents: Whole Blood and Plasma Derivatives, Blood **5:** 401-420, 1950.
13. Dreskin, O. H., and Rosenthal, N.: A Hemophilia-like Disease With Prolonged Coagulation Time and a Circulating Anticoagulant, Blood **5:** 46-60, 1950.
14. Van Creveld, S., Hoorweg, P. G., and Paulssen, M. M. P.: Research on a Circulating Anticoagulant in a Hemophiliac, Blood **6:** 233-241, 1951.
15. Quick, A. J.: Studies on the Enigma of the Hemostatic Dysfunction of Hemophilia, Am. J. M. Sc. **214:** 272-280, 1947.
16. Quick, A. J., and Hussey, C. V.: Hemophilia: Clinical and Laboratory Observations Relative to Diagnosis and Inheritance, Am. J. M. Sc. **223:** 401-413, 1952.
17. Fantl, P., and Nance, M. H.: An Acquired Hemorrhagic Disease in a Female Due to an Inhibitor of Blood Coagulation, M. J. Australia **2:** 125-129, 1946.
18. Heinle, R. W., Weisberger, A. S., Vignos, P. J., and Holden, W. B.: Hemorrhagic Diathesis Associated With Low Thromboplastic Activity and a Circulating Anticoagulant, J. Lab. & Clin. Med. **34:** 1606-1607, 1949.

19. Collins, I. S., and Ferriman, D. G.: Haemophilia-like Syndrome from Anticoagulants Affecting the Production of Thromboplastin, Lancet **2**: 712-718, 1952.
20. Hougie, C.: Pseudohaemophilia: An Acquired Haemorrhagic Diathesis Due to a Circulating Anticoagulant, J. Clin. Path. **6**: 30-33, 1953.
21. Van Creveld, S., Hoorweg, P. G., and Paulssen, M. M. P.: Researches on a Circulating Anticoagulant in a Hemophiliac. II. Effect of Administration of ACTH and Cortisone, Blood **8**: 125-130, 1953.

THE HEMODYNAMIC AND METABOLIC RESPONSE OF THE HUMAN HYPERTENSIVE KIDNEY TO A STANDARD DOSE OF 1-HYDRAZINOPHTHALAZINE (HYDRALAZINE)

ARCHER P. CROSLEY, JR., M.D., GEORGE G. ROWE, M.D.,* AND CHARLES W. CRUMPTON, M.D., MADISON, WIS.

PREVIOUS observations by Barker and associates[1] in normotensive subjects demonstrated that the increase in renal blood flow, produced by the intravenous administration of salt-poor human albumin, resulted in a disproportionate decrease in arterial-renal venous oxygen difference, with a significant reduction in oxygen consumption. Since Hydralazine has been shown to increase renal blood flow in hypertensive patients,[2] this agent appeared to be ideal for the investigation of the interrelationships between the vascular and metabolic reactivity of the hypertensive kidney.

The purpose of this paper is to present the renal metabolic and vascular adjustments following the intravenous administration of 0.25 mg. per kilogram of Hydralazine to hypertensive patients.

MATERIALS AND METHODS

Six fasting, hypertensive patients of Smithwick grade II to IV were studied for two fifteen-minute periods before (C) and two fifteen-minute periods (30 to 45 an 45 to 60 minutes) after (E) the intravenous administration of 0.25 mg. per kilogram of 1-hydrazinophthalazine (Hydralazine).† The selection of these times was based on statistical design and the previous observation in this laboratory that blood pressure stabilization, following the intravenous use of this agent, occurred between 30 and 60 minutes after injection.[3]

In four such subjects, measurements of glomerular filtration rate (inulin clearance), total renal blood flow (Fick PAH), and arterial-renal venous oxygen differences were determined, utilizing routine clearance techniques[4] and right renal vein catheterization. Oxygen consumption was calculated as the product of total renal blood flow and arterial-renal venous oxygen difference. In the remaining two individuals, these measurements were supplemented by the determination of the tubular maximal secretory capacity of PAH. In one of the six patients, data on cardiac output (Fick) were collected just prior to, and at the completion of, the renal study. Blood pressure was recorded by means of an indwelling intra-arterial needle utilizing a strain gauge manometer and a Sanborn Poly-Viso recorder.

Analyses of inulin and PAH in plasma and urine were performed according to the methods of Harrison[5] and Smith and colleagues,[6] respectively.

From the Cardiovascular Laboratory and The Department of Medicine of The University Hospitals, University of Wisconsin and The Veterans Administration Hospital, Madison, Wis.

This work was supported by grants from the National Heart Institute, United States Public Health Service, the Wisconsin Heart Association and the Wisconsin Alumni Research Foundation.

Received for publication, March 4, 1954.

*Research Fellow of The American Heart Association.

†Supplied by Ciba Pharmaceutical Products, Inc., Summit, N. J.

Determinations of oxygen were carried out by the manometric method of Van Slyke and Neill[7] on whole blood samples collected anaerobically in heparinized syringes. Duplicate runs on arterial and renal venous oxygen contents checked within ± 0.05 volume per cent. Although measurements of oxyhemoglobin were made on the individual samples to ascertain factors of dilution caused by the presence of the anticoagulant, the results are uncorrected for this factor, since differences in the corrected and uncorrected values were not statistically significant. In addition, the variability of the oxyhemoglobin among repeated samples of the same specimen of blood was not significantly different from the variability among any given arterial-renal venous difference ($2\sigma = \pm 0^2$ scale divisions $= \pm$ 0.2 vol. per cent). Such variability is within the error of the instrument (Coleman Senior Spectrophotometer).[8]

Hematocrits were determined by the method of Wintrobe,[9] uncorrected for trapped plasma.

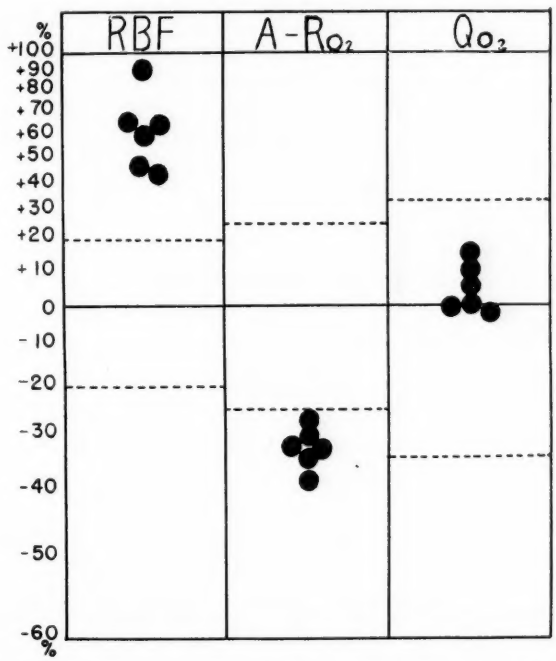

Fig. 1.—Effect of 0.25 mg. per kilogram of Hydralazine on renal metabolism. Circles represent percentage change from mean control levels indicated by the horizontal solid line at O. Dotted horizontal lines represent ± 2.47 standard errors of measurement for each function or the limits of statistical significance when $p = < .05$ $n = 6$. Abbreviations same as in Table I.

RESULTS

The results are summarized in Table I. Fig. 1 is a diagrammatic summary of the metabolic observations.

The intravenous administration of 0.25 mg. per kilogram of Hydralazine produced a statistically significant reduction (–19 per cent) in mean arterial blood pressure ($p = < .01$). The latter was accompanied by a significant and disproportionately greater increase in total renal blood flow (+62 per cent $p = < .01$) caused by a reduction in renal peripheral resistance (–47 per cent $p = < .01$). Glomerular filtration rate remained unchanged.

TABLE I

PATIENT	PERIOD	MABP[3] MM. Hg	% Δ[4]	RBF[5] ML./MIN.	% Δ	RPR[6] A.U.[7]	% Δ	GFR[8] ML./MIN.	% Δ	A-Ro_2[9] VOL. %	% Δ	Qo_2[10] ML./MIN.	% Δ	E_{PAH}[11] %	% Δ	Tm_{PAH}[12] Mg./MIN.	% Δ	C.O.[13] L./MIN.	% Δ
W.K.	C[1]	108		100		1.1		6		2.1		2.1		(31)					
34-W-F														33					
SA = 1.45	E[2]	85	-23*	165	+65*	0.52	-55*	6	0	1.3	-38*	2.1	0	(20)	-39*				
														20					
E.S.	C	170		460		0.37		48		2.2		10.0		33		44			
41-W-M																			
SA = 1.99	E	152	-19*	660	+44*	0.23	-38*	43	-10	1.5	-32*	9.9	- 2	21	-36*	45	+2		
M.J.	C	120		985		0.12		96		1.0		9.9		90					
53-W-M																			
SA = 2.05	E	105	-18*	1575	+60*	0.07	-42*	96	0	0.7	-30*	11.0	+11	85	- 5				
H.B.	C	142		560		0.25		75		1.8		10.0		87					
45-W-F																			
SA = 1.30	E	122	-13*	970	+90*	0.13	-48*	82	+10	1.2	-33*	11.6	+16	89	+ 2				
V.B.	C	172		875		0.20		65		1.8		15.7		31		76			
42-W-F																			
SA = 1.66	E	135	-21*	1200	+45*	0.11	-45*	60	- 7	1.3	-28*	15.6	0	20	-35*	70	-8		
E.L.	C	156		470		0.33		66		2.0		9.4		93				6.8	
44-W-F																			
SA = 1.42	E	122	-22*	775	+65*	0.16	-51*	72	+ 9	1.3	-35*	10.1	+ 7	90	- 3			7.0	+3
Mean % Δ[14]			-19*		+62*		-47*		0		-33*		+ 5		-20*		-6		+3
$o\bar{x}$[15]			± 3.7		± 8.2		± 3.3		± 6.6		±10.2		±14.1		± 3.9				

*Denotes statistical significance p = < .05. () = Whole blood extraction.

[1]C = Average of two control periods.
[2]E = Average of two experimental periods.
[3]MABP = Mean arterial blood pressure.
[4]% Δ = Per cent change from mean control value.

[5]RBF = Total renal blood flow = $\frac{UV_{PAH}}{\frac{A_{PAH} - R_{PAH}}{1 - Hct}}$

[6]RPR = Total peripheral resistance = $\frac{MABP}{RBF}$

[7]Arbitrary Units.
[8]GFR = Glomerular filtration rate.
[9]A-Ro_2 = Arterial-renal venous oxygen difference.
[10]Qo_2 = Oxygen consumption = RBF × A-Ro_2

[11]Extraction PAH = $\frac{A_{PAH} - R_{PAH}}{A_{PAH}}$

[12]Tm_{PAH} = Tubular maximal secretory capacity of PAH.
[13]C.O. = Cardiac output (Fick).
[14]Mean % Δ = Mean change of group from individual mean control values.
[15]$o\bar{x}$ = Standard error of measurement expressed as per cent.

The enhancement of renal blood flow was associated with a proportional reduction in arterio-renal venous oxygen difference (–33 per cent $p = < .01$) such that the product of these two factors, namely, the renal oxygen consumption, remained essentially unchanged.

Plasma extraction of PAH decreased significantly in three cases. A further check of whole blood extraction in one of these cases demonstrated that this fall was not the result of an altered ratio between the plasma and red blood cells. In two patients the tubular maximal secretory capacity of PAH remained unchanged.

In one case, the simultaneous measurement of cardiac output demonstrated essentially no change in this function despite a 65 per cent increase in total renal blood flow.

DISCUSSION

The results in these six patients demonstrate that the hypertensive kidney, in response to an increased renal blood flow produced by a standard intravenous dose of Hydralazine, is capable of decreasing its arterial-renal venous oxygen extraction, thereby maintaining its initial level of oxygen consumption. Thus the maintenance of Q_{O_2} is a different result from that previously reported in normotensive individuals when renal blood flow was increased by salt-poor human albumin.[1] The explanation for such a difference is not apparent. Two possibilities present themselves. The observation in normotensive individuals[1] might have been related to an effect of the albumin per se on the metabolic pattern. On the other hand, the maintenance of oxygen consumption in these hypertensive patients might reflect a loss of renal metabolic reactivity. Since the pattern described following Hydralazine is similar to that found in other organs of the body in response to an increased regional blood flow,[12] the first explanation would seem the more plausible one.

The finding of a decreased PAH extraction in three patients is in accord with the observations of Dustan and associates,[10] and might suggest the presence of intrarenal vascular shunts. The maintenance of glomerular filtration rate and Tm_{PAH} indicate, however, that if such shunting occurs it is in addition to, rather than to the exclusion of, previously functioning channels. Furthermore, the fact that the metabolic pattern remained the same whether or not a decrease in E_{PAH} occurred, precludes the possibility that the changes in arterial-renal venous oxygen difference are primarily related to vascular shunting and arterialization of the renal venous blood. These data lend additional support to the concept that the human kidney, like other body organs, is capable of altering its arterial-renal venous oxygen difference in response to changes in renal blood flow. This is in contrast to previous reports that the oxygen consumption of the kidney is a direct function of alterations in renal blood flow.[11]

One of the patients studied showed an increase in cardiac output of only +3 per cent at a time when renal blood flow was enhanced +65 per cent. This led to a comparison of the results obtained in these six patients with a similar series of fourteen hypertensive patients[12] studied in this laboratory in whom measurements of cardiac output were made before and after a similar dose of

Hydralazine. Such a comparison led to the interpretation that in the hypertensive subject, Hydralazine produces a mean increase in renal blood flow (+62 per cent) out of proportion to the mean increase in cardiac output (+28 per cent). The difference between these two figures is highly significant ($p = < .01$). Thus the relationship between renal blood flow and cardiac output in hypertensive patients would appear to be the reverse of that previously reported by Wilkinson and associates[13] in normotensive individuals.

SUMMARY AND CONCLUSIONS

Hydralazine was administered to six hypertensive patients in a standard dose of 0.25 mg. per kilogram. Associated with a significant decrease in mean arterial blood pressure (–19 per cent), an increase in renal blood flow (+62 per cent) occurred, caused by a marked reduction in renal peripheral resistance (–47 per cent). The enhancement of renal blood flow was accompanied by a decrease in arterial-renal venous oxygen difference such that the renal oxygen consumption was maintained. A comparison of the increase in renal circulation with measurements of cardiac output suggests that the hypertensive kidney receives a disproportionately greater quantity of blood than might be predicted from the change in cardiac output. The maintenance of glomerular filtration rate and tubular maximal secretory capacity of PAH despite a decrease in arterial-renal venous oxygen difference and a diminished extraction of PAH suggest that if intravascular shunts occur, they are in addition to, rather than to the exclusion of, previously existing vascular channels.

The authors wish to acknowledge the valuable technical assistance provided by Mrs. Marion Hindersinn, Miss Mary Novitski, Miss Beryl Welch, Mrs. Audrey Peterson and Mrs. Karine Hovey.

REFERENCES

1. Barker, H. G., Clark, J. K., Cummins, A. J., and Crosley, A. P., Jr.: Unpublished observations.
2. Reubi, F. C.: Renal Hyperemia Induced in Man by a New Phthalazine Derivative, Proc. Soc. Exper. Biol. & Med. **73**: 102, 1950.
3. Rowe, G. G., Huston, J. H., Maxwell, G. M., Crosley, A. P., Jr., and Crumpton, C. W.: Unpublished observations.
4. Goldring, W., and Chasis, H.: Hypertension, New York, 1944, The Commonwealth Fund.
5. Harrison, H. E.: A Modification of the Diphenylamine Method for the Determination of Inulin, Proc. Soc. Exper. Biol. & Med. **49**: 111, 1942.
6. Smith, H. W., Finklestein, N., Aliminosa, L., Crawford, B., and Graber, M.: The Renal Clearance of Substituted Hippuric Acid Derivatives and Other Aromatic Acids in Dogs and Man, J. Clin. Investigation **24**: 388, 1945.
7. Van Slyke, D. D., and Neill, J. M.: The Determination of Gases in Blood and Other Solutions by Vacuum Extraction and Manometric Measurement, J. Biol. Chem. **61**: 523, 1924.
8. Operating Directions for the Coleman Model 14 Universal Spectrophotometer: Maywood, Ill., 1947.
9. Wintrobe, M. M.: A Simple and Accurate Hematocrit, J. Lab. & Clin. Med. **15**: 287, 1929.
10. Dustan, H. P., Corcoran, A. C., Taylor, R. D., and Page, I. H.: Renal Vascular Response to Intravenous 1-Hydrazinophthalazine in Relation to Therapeutic Responses to Chronic Oral Dosage, Proc. Am. Fed. Clin. Res., 10th Annual Meeting of The Midwestern Section **11**: 1952.

11. Van Slyke, D. D., Rhoads, C. P., Hiller, A., and Alving, A. S.: Relationships Between Urea Excretion, Renal Blood Flow, Renal Oxygen Consumption, and Diuresis. The Mechanism of Urea Excretion, Am. J. Physiol. **109**: 336, 1934.
12. Crumpton, C. W., Rowe, G. G., Crosley, A. P., Jr., Maxwell, G. M., and Huston, J. H.: Cardiovascular, Cerebral and Renal Hemodynamics and Metabolic Adjustments to 1-Hydrazinophthalazine in Essential Hypertension, J. LAB. & CLIN. MED. **42**: 797, 1953.
13. Wilkinson, E. L., Beckman, H., and Hecht, H. H.: Cardiovascular and Renal Adjustments to a Hypertensive Agent (1-Hydrazinophthalazine: Ciba BA-5968: Apresoline), J. Clin. Investigation **31**: 872, 1952.

HUMAN SERUM ALBUMIN TAGGED WITH I^{131} IN PATIENTS WITH ASCITES CAUSED BY ABDOMINAL CARCINOMATOSIS AND PORTAL CIRRHOSIS: THE RATES OF INTERCHANGE BETWEEN THE VASCULAR COMPARTMENT AND PERITONEAL CAVITY

MALCOLM P. TYOR, M.D.
OAK RIDGE, TENN.

INTRODUCTION

SINCE Von Recklinghausen first described the cells lining the serous surfaces, many studies have dealt with the transfer of various materials between the peritoneal cavity and vascular compartment.[1] In most of these investigations, various types of particulate matter and solutions were injected into the peritoneal cavity of normal animals; observations were then made on the mode of transfer and time of appearance of such substances in the blood and lymphatics.[1-4]

In previous clinical investigations, measurements have been made of colloid osmotic pressure and albumin concentration in the plasma and ascitic fluid of patients with cirrhosis, after the intravenous injection of relatively large amounts of concentrated albumin.[5, 6]

In the present study, 5 to 10 mg. of human serum albumin labeled with I^{131} was injected intravenously or intraperitoneally into patients with ascites caused by abdominal carcinomatosis or portal cirrhosis. The rates of decreasing concentration of the tagged albumin from the injected compartment and increasing concentration in the other were determined.

MATERIALS AND METHODS

Subjects.—Eleven hospitalized women with ascites were studied; nine had abdominal carcinomatosis, and two had portal cirrhosis. All diagnoses were proved by biopsy. The patients with carcinomatosis all had normal serum protein concentrations and albumin-globulin values; none had clinical evidence of cirrhosis, nor significant alterations in the several liver function tests* performed. Both cirrhotic patients had relatively low plasma albumin values and abnormal liver function. Proteinuria was not present in any patient.

All patients had been requiring paracenteses at ten-day to three-week intervals. Paracenteses, with the aspiration of an appreciable amount of fluid, were not performed for at least five days prior to the study nor during the study. All subjects showed evidence of ascitic fluid accumulation during the period of observation, and an individual gain in body weight of 0.5 to 2.3 kg. Since their general condition was poor, and most had an impaired food intake, the increasing body weight probably failed to indicate the true rate of accumulation of fluid.

I^{131}-Tagged Albumin.†—Each lot of iodinated albumin used in the present study was dialyzed at 4° C. under sterile conditions for eighteen to forty-eight hours against distilled water in beakers changed at frequent intervals. At the end of the dialysis, the radioactivity

Work done at the Oak Ridge Institute of Nuclear Studies, Medical Division, Oak Ridge, Tenn., under contract with the United States Atomic Energy Commission.

Received for publication, March 5, 1954.

*Bromsulfalein retention, van den Bergh's, cephalin cholesterol flocculation, thymol turbidity, and urine urobilinogen.

†The tagged serum albumin was obtained from the Department of Radioactive Pharmaceuticals, Abbott Laboratories, North Chicago, Ill.

outside the membrane was between 1 and 3 per cent of the total; 90 per cent of this dialyzable activity appeared in the first eight hours.

Immediately following the dialysis, and just prior to administration, the iodinated albumin was diluted with sterile physiologic saline solution to give 20 to 30 μc per milliliter of solution to be injected.

Route and Method of Injection.—Five patients with carcinomatosis were given the tagged albumin intraperitoneally; in four the material was administered by the intravenous route. The tagged albumin was injected intraperitoneally into one patient with cirrhosis and intravenously into the other.

In the patients injected by the intraperitoneal route, a polyethylene tube was inserted into the peritoneal cavity through a 15-gauge needle, which was subsequently withdrawn. The tubing was held in place by a purse-string suture. Ten milliliters of the iodinated albumin solution were injected through a calibrated syringe directly into the peritoneal cavity at a site on the opposite side of the abdomen from that of the polyethylene tubing. The syringe was rinsed several times with ascitic fluid. The patient was then turned in various positions, and the abdomen massaged in order to facilitate mixing. In each patient, six samples, each consisting of 10 ml. of ascitic fluid, were withdrawn through the polyethylene tubing at ten-minute intervals and were treated with heparin. The injected tagged albumin was uniformly distributed throughout the ascitic fluid within twenty minutes after injection, and at sixty minutes showed no significant variation. The volume of ascitic fluid was calculated by dividing the total radioactivity injected by the concentration after complete mixing.

Ten milliliter samples of ascitic fluid and blood were then obtained concomitantly and heparinized at 3, 6, 12, 24, 48, 72, 96, and 120 hours after injection. All samples were centrifuged at 3,500 r.p.m. for twenty minutes. The plasma and supernatant ascitic fluid were transferred to clean, dry test tubes and were then stored for counting and determination of the albumin concentration.

In those patients injected intravenously, the polyethylene tube was inserted into the mid-lower portion of the peritoneal cavity in a manner similar to that described previously. Following this procedure, 10 ml. of the iodinated albumin solution was injected intravenously through a sterile calibrated syringe. Ten milliliters of blood were then obtained fifteen minutes later from the arm opposite to the site of injection and was treated with heparin, this sample being used for determination of plasma volume.[7] Venous and ascitic samples were obtained at the same time intervals as in those patients injected intraperitoneally and were treated similarly.

Counting Methods.—Gamma counting, using a Marinelli cup and bismuth-walled Geiger-Müller tube, was performed on all samples.[8] The activity of all samples was such that the probable counting error was less than 3 per cent.

In several samples, taken at various time intervals in both groups of patients, 1 ml. each of both plasma and ascitic fluid was precipitated with 4 ml. of 10 per cent trichloroacetic acid. The total activity in 1 ml. was compared with the activity in the trichoroacetic acid precipitate. Beta counting was employed, using an end window Geiger-Müller tube.[8] Ninety to 95 per cent of the activity was present in the trichloroacetic acid precipitate.

Stable Methods.—The albumin was measured photometrically by the biuret reaction after precipitation of the globulin with 22 per cent sodium sulfate.

RESULTS

The initial volumes of fluid in the compartments of injection in all patients are shown in Table I, along with the concentration of albumin in the ascitic fluid and plasma, and the total content of albumin in the two compartments of injection. Since the concentrations of stable albumin in the blood and ascitic fluid in each patient showed no significant change during the study, the mean values are shown.

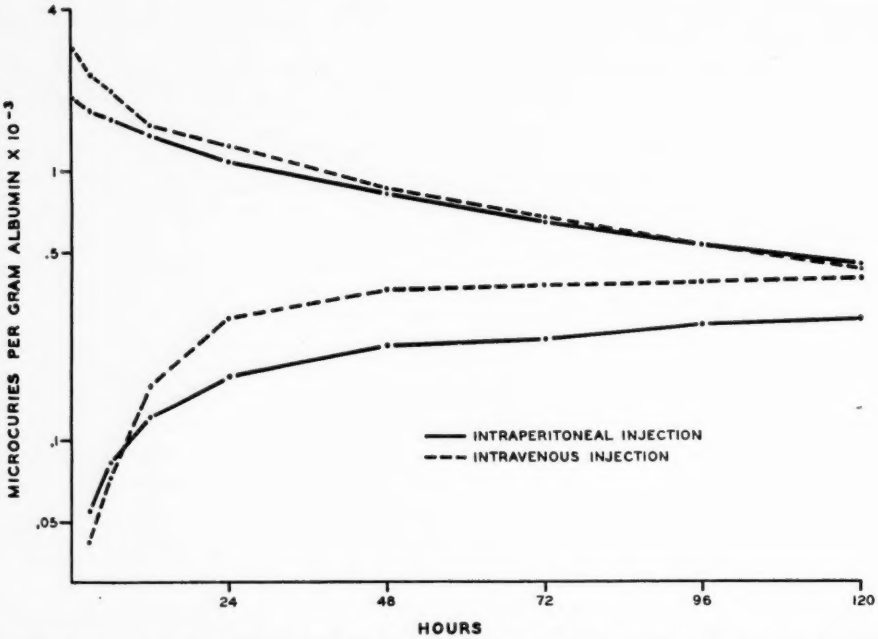

Fig. 1.—Rate of exchange of iodinated serum albumin between the vascular compartment and peritoneal cavity. Carcinomatosis with ascites; mean values in two groups of patients. Semilogarithmic plot. The descending unbroken and broken curves represent the mean values of decline in concentration of radioactivity from the ascitic fluid and plasma in the patients with carcinoma. The ascending curves represent the corresponding increase in concentration of radioactivity in the opposite compartments.

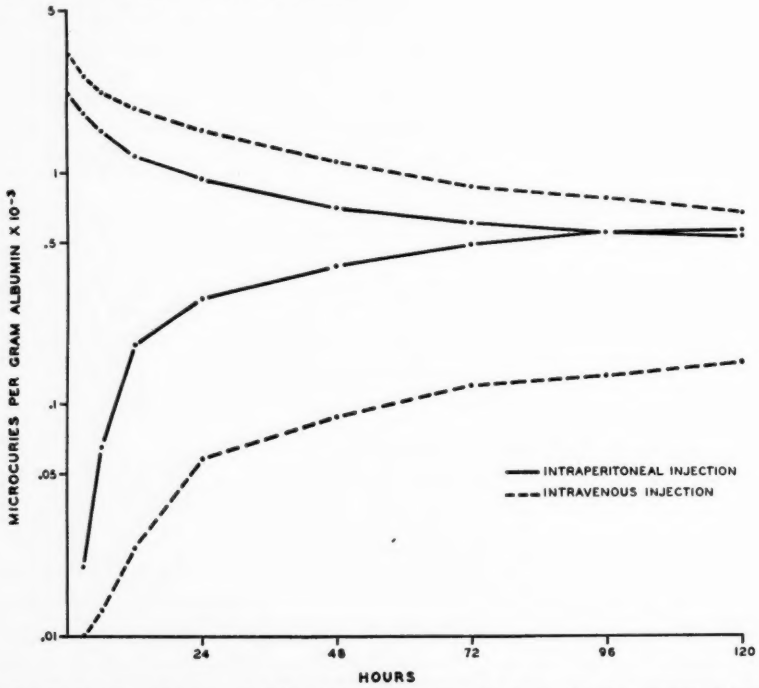

Fig. 2.—Rate of exchange of iodinated serum albumin between the vascular compartment and peritoneal cavity. Cirrhosis with ascites; two patients. Semilogarithmic plot. The descending unbroken and broken curves represent the decline in concentration of radioactivity from the ascitic fluid and plasma in two patients with cirrhosis. The ascending curves represent the corresponding increase in concentration of radioactivity in the opposite compartments.

TABLE I. THE INTERCHANGE OF TAGGED ALBUMIN BETWEEN THE PLASMA AND ASCITIC FLUID AFTER INTRAPERITONEAL AND INTRAVENOUS INJECTION

ROUTE OF INJECTION	DISEASE	PATIENT	ASCITIC FLUID VOLUME (ML.)*	ALBUMIN CONCENTRATION (GM. %)†		ALBUMIN CONTENT ASCITIC FLUID (GM.)‡	RATE OF INTERCHANGE $T\frac{1}{2}$ (HOURS)§
				ASCITIC	PLASMA		
Intraperitoneal	Carcinoma with ascites	J. G.	4,359	2.85	4.00	124	39
		A. M.	2,409	3.14	4.01	76	11
		D. H.	3,520	3.42	4.00	120	52
		M. G.	2,709	3.97	4.47	108	51
		M. C.	3,408	2.96	3.83	101	51
	Cirrhosis with ascites	M. S.	3,158	2.98	3.41	94	17
			PLASMA VOLUME (ML.)*			ALBUMIN CONTENT PLASMA (GM.)‡	
Intravenous	Carcinoma with ascites	R. E.	1,301	3.36	4.11	53	18
		B. S.	2,189	3.42	4.30	94	66
		M. A.	2,081	3.54	3.96	82	46
		L. M.	4,768	3.38	4.10	195	26
	Cirrhosis with ascites	L. H.	2,908	2.63	3.17	92	64

*Initial volume of injected compartment.

†Mean values for individual determinations throughout five days of study (mean difference for individual determinations: 0.05 to 0.21 Gm. per cent).

‡Albumin content of injected compartment at beginning of study.

§Rate of interchange is herein defined as the slope of the line obtained from the differences between the specific activities at the various time intervals ($t\frac{1}{2}$ = half-time).

In the determination of the rates of disappearance of the tagged albumin from the injected compartment and its appearance in the other, the values of the various plasma and ascitic fluid samples were expressed in terms of specific activity (microcuries per gram of albumin) (Figs. 1 and 2).

Behavior of Tagged Albumin in the Injected Compartment.—In all patients, following the administration of iodinated albumin into either peritoneal fluid or blood, a rapid initial decline in the concentration of radioactivity occurred in the injected cavity within the first twelve to twenty-four hours. This early fall in activity was greater in the patients with carcinomatosis injected intravenously than in those given intraperitoneal injections. After twelve to twenty-four hours, there was a more gradual rate of disappearance from the injected compartment with both routes of injection. The slopes of the curves showing disappearance in the two compartments were similar throughout the remainder of the 120 hours of observation (Fig. 1). In general, the curves of disappearance of tagged albumin in the two cirrhotic patients were similar to those seen in individual cases of carcinomatosis (Fig. 2).

Appearance in the Other Compartment.—A significant amount of radioactivity appeared within three hours in the vascular compartment after the injection of tagged albumin into the peritoneal cavity, and in the peritoneal cavity after its intravenous injection. In all patients there was a relatively rapid increase in activity during the first twenty-four hours after injection. In those with carcinomatosis, this increase was somewhat more pronounced in the patients given the albumin intravenously. The increase in radioactivity was more gradual after twenty-four to forty-eight hours with either route of injection in the two diseases, and during the remainder of the study the curves showed similar slopes of appearance (Figs. 1 and 2).

Equilibrium Time.—The level of specific activity of albumin in the injected compartment and in the other approached an equilibrium in all patients studied. Complete equilibrium between the two compartments was not attained in four of the patients with carcinomatosis to whom the albumin was administered intraperitoneally. In the other patient, uniform distribution of the iodoalbumin throughout the two compartments occurred within fifty-eight hours after injection. In those patients with carcinomatosis who were given intravenous injections, equilibrium was not reached in two during the period of study. In the two other patients, uniform distribution between the two compartments occurred at approximately 108 hours (Fig. 3) and 118 hours, respectively. Equilibrium time in the two cirrhotic patients was approached at a rate similar to those with carcinomatosis.

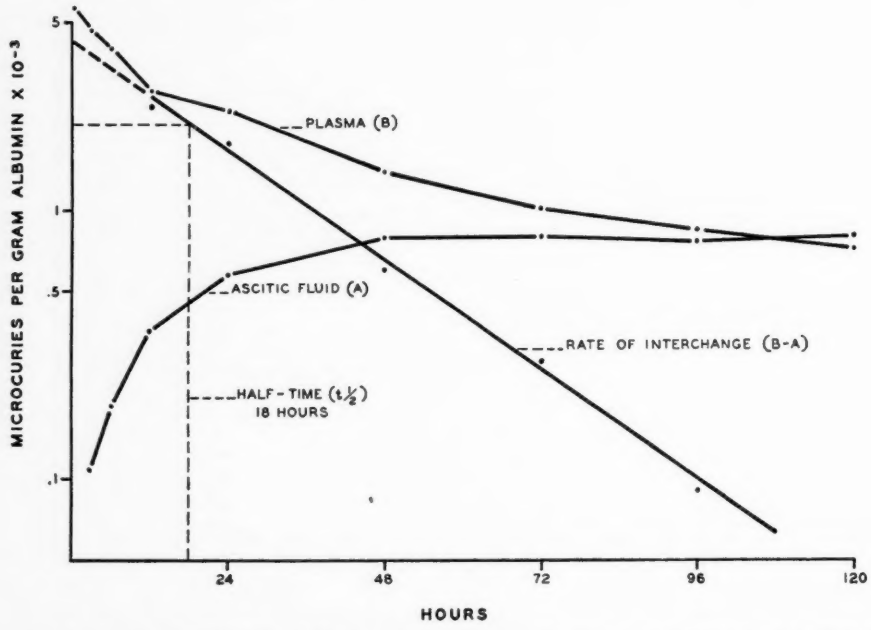

Fig. 3.—Rate of interchange of I^{131}-tagged albumin between the plasma and ascitic fluid. Abdominal carcinomatosis (Patient R. E.). Semilogarithmic plot. In this subject, uniform distribution of the intravenously administered tagged albumin between the plasma and ascitic fluid occurred at approximately 108 hours. The linear plot (rate of interchange) was obtained from the difference between the specific activities of the plasma and ascitic fluid at the specified time intervals. The half-time ($t\frac{1}{2}$) was obtained graphically as shown by the horizontal and vertical dotted lines.

Rate of Interchange.—With either route of injection, the difference between the specific activities of the two compartments, at each of the observed intervals, appeared as a straight line when plotted on a semilogarithmic scale against time. Since the specific activity of the tagged albumin approached a common level in both compartments, and since there was continual interchange between the two, the slope of the linear plot was considered to represent a measure of the rate of interchange of the administered iodinated albumin in the two compartments (Fig. 3). The half-time can be read graphically from the slope of the linear plot; individual values are shown in Table I.

COMMENT

It is apparent in these patients with carcinomatosis and cirrhosis that albumin is constantly passing into and out of both plasma and ascitic fluid, in conformance with the concept that the membrane between the vascular and peritoneal compartments is permeable to albumin and that a dynamic state of exchange exists.

Although Berson and his group have recently indicated that in vitro labeling of albumin with iodine may induce alterations in the metabolic degradation rate of the albumin molecule, it is probable that its behavior in distribution phenomena closely reflects that of native albumin, since ultracentrifugal and electrophoretic analyses of the two are indistinguishable.[9, 10] Similar exchange patterns of tagged albumin between plasma and ascitic fluid have been recorded in the dog, using protein labeled in vivo with carbon 14.[11]

Following the injection of iodine-labeled albumin into either the blood or ascitic fluid, the apparent equilibrium indicated by the convergence of the curves of appearance and disappearance represents the time necessary for the tagged albumin to become completely mixed with the plasma and ascitic fluid protein. This apparent equilibrium does not represent a transfer of equal quantities of protein in opposite directions, since the amount of ascitic fluid was continually increasing in all patients studied.

Uniform concentrations of the tagged albumin between the two compartments were attained in two patients following intravenous injection and in two patients following intraperitoneal injection, during the period of study. In these cases the measure for half-time of interchange reflected the relatively rapid equilibrium point. In patients in whom equilibrium time appeared to be prolonged, such as in the cirrhotic patient injected intravenously, the half-time was increased. It was not possible to delay paracentesis in many of the patients studied. In such patients, the measurement for half-time of interchange served to shorten the period of observation and still allowed approximation of equilibrium time between compartments.

The fluid volume and concentration of albumin in blood and ascitic fluid have been shown to influence the rate of transfer of water and protein.[5] In the present study, there was no consistent relationship between the half-time of interchange in individual patients and the initial volume of fluid or concentration of albumin in the injected compartment; nor was there any consistent relationship between this approximation of equilibrium time and the rate of ascitic fluid accumulation, as judged by the frequency of required paracenteses.

Prentice and associates[12] have studied the exchange of tritium-labeled water between the peritoneal cavity and vascular compartment in patients with carcinomatosis and cirrhosis; here, in both diseases, uniform concentration between compartments may occur within ten hours. In the present study, using a protein of relatively high molecular weight, equilibrium time was comparatively prolonged in the patients with carcinoma and in those with

cirrhosis. In dogs with experimental ascites, globulin as well as albumin has been shown to exchange between the plasma and ascitic fluid; uniform concentration of globulin between compartments was somewhat slower, however, than that of albumin.[11] Although there are many individual factors which influence the complex disturbance of ascitic fluid accumulation, these studies suggest that the ability to differentiate between molecular size is maintained and that similar mechanisms are at play in the two diseases studied.

Regardless of cause, ascitic fluid accumulation must be due to an imbalance between inflow and outflow from the peritoneal cavity; either or both factors could be responsible.[12] A decrease in ascitic fluid accumulation is frequently evident following various forms of therapy. It is suggested that the measurement for half-time of interchange of tagged albumin may reflect the over-all defect in the mechanisms causing ascitic fluid accumulation and that repeated determinations in the same patient, before and after treatment, may help to clarify these problems.

SUMMARY

Tracer amounts of human serum albumin labeled with I^{131} were injected intravenously or intraperitoneally into nine patients with abdominal carcinomatosis and ascites. The rates of decreasing concentration of the tagged albumin in the injected compartment and increasing concentration in the other were similar after the initial twelve to twenty-four hours. In two patients with cirrhosis and ascites, the patterns of exchange between compartments were similar to those seen in patients with carcinoma. The specific activity of albumin in the injected compartment and in the other approached a common value in all subjects studied. Complete equilibrium of the injected tagged albumin between compartments occurred in four patients during the period of study. Equilibrium time between compartments was approximated from the slope of the linear plot obtained from the differences between the specific activities of the plasma and ascitic fluid. The half-times were found to be eleven to fifty-two hours for intraperitoneal injection and eighteen to sixty-six hours for intravenous injection.

REFERENCES

1. Cunningham, R. S.: The Physiology of the Serous Membranes, Physiol. Rev. **6**: 242-280, 1926.
2. MacCallum, W. G.: On the Mechanism of Absorption of Granular Materials From the Peritoneum, Bull. Johns Hopkins Hosp. **14**: 105-115, 1903.
3. Siperstein, D. M., and Sansby, J. M.: Intraperitoneal Transfusion With Citrated Blood. An Experimental Study, Am. J. Dis. Child. **25**: 107-129, 1923.
4. Hahn, P. F., Miller, L. L., Robscheit-Robbins, F. S., Bale, W. F., and Whipple, G. H.: Peritoneal Absorption. Red Cells Labelled by Radio-Iron Hemoglobin Move Promptly From Peritoneal Cavity Into the Circulation, J. Exper. Med. **80**: 77-82, 1944.
5. Mankin, H., and Lowell, A.: Osmotic Factors Influencing the Formation of Ascites in Patients With Cirrhosis of the Liver, J. Clin. Investigation **27**: 145-153, 1948.
6. Kunkel, H. G., Labby, D. H., Ahrens, E. H., Jr., Shank, R. E., and Hoagland, C. L.: The Use of Concentrated Human Serum Albumin in the Treatment of Cirrhosis of the Liver, J. Clin. Investigation **27**: 305-319, 1948.

7. Tyor, M. P., and Cayer, D.: The Rate of Disappearance of Iodinated Human Albumin From the Serum in Relation to Albumin Concentration, Total Circulating Albumin Content, and the Presence of Ascites in Patients With Cirrhosis, J. LAB. & CLIN. MED. **39:** 874-881, 1952.
8. Bruner, H. D., and Perkinson, J. D., Jr.: A Comparison of Iodine^{131} Counting Methods, Nucleonics **10:** 57-61, 1952.
9. Berson, S. A., Yalow, R. S., Schreiber, S. S., and Post, J.: Tracer Experiments With I^{131} Labelled Human Serum Albumin: Distribution and Degradation Studies, J. Clin. Invest. **32:** 746-768, 1953.
10. Sterling, K.: The Turnover Rate of Serum Albumin in Man as Measured by I^{131}-Tagged Albumin, J. Clin. Invest. **30:** 1228-1237, 1951.
11. McKee, F. W., Yuile, C. L., Lamson, B. G., and Whipple, G. H.: Albumin and Globulin Circulation in Experimental Ascites. Relative Rates of Interchange Between Plasma and Ascitic Fluid Studied With C 14-Labelled Proteins, J. Exper. Med. **95:** 161-172, 1952.
12. Prentice, T. C., Siri, W., and Joiner, E. E.: Quantitative Studies of Ascitic Fluid Circulation With Tritium-Labelled Water, Am. J. Med. **13:** 668-673, 1952.

THE RELATIONSHIP OF VITAMIN B_{12} AND FOLIC ACID IN MEGALOBLASTIC ANEMIAS

H. O. Nieweg, M.D., J. G. Faber, Ph.D., J. A. de Vries, and W. F. Stenfert Kroese
Groningen, Netherlands

SINCE the introduction of liver therapy by Minot and Murphy, megaloblastic anemia has generally been recognized as a manifestation of deficiency disease.

During the past decade, a number of substances have been found to have therapeutic activity in megaloblastic anemia. These factors can be divided in three groups:

1. Factors of the folic acid group: pteroylglutamic acid, citrovorum factor (folinic acid), and their conjugates. In this study, we use the term folic acid for factors stimulating the growth of *Streptococcus faecalis* (including, therefore, CF and PGA).

2. Factors of the vitamin B_{12} group: the different cobalamines.

3. Constituents of nucleic acids: uracil, thymine, and thymidine.[1-6]

From the clinical point of view, vitamin B_{12} and folic acid are the most important factors. We have estimated the vitamin B_{12} level in the serum, and the folic acid activity in the blood of a number of normal persons and patients with different types of megaloblastic anemia. A review of the interrelationship of these factors is presented.

METHODS

Vitamin B_{12} Determination in Serum.—We used *Lactobacillus leichmannii* A.T.C.C. 7830 Strain 313. The culture was carried twice a week into stock culture in a medium of the following composition:

Glucose-vitamin mixture	2.5 Gm.
$CaCO_3$	2.5 Gm.
Agar	2.5 Gm.
Yeast extract	2.0 Gm.
Vitamin B_{12}	150.0 μg
Crude liver extract (20 Gm. per ml.)	2.5 ml.
Water to	100.0 ml.

The glucose-vitamin mixture we used for the preparation of the basal medium consisted of the following composition:

Glucose	800 Gm.
Tryptophan	4 Gm.
Cystine	4 Gm.
Adenine	200 mg.
Guanine	200 mg.
Uracil	200 mg.
Xanthine	200 mg.

From the Department of Internal Medicine (Chief: Prof. F. S. P. van Buchem, M.D.), State University of Groningen, and the Microbiological Laboratory, N. V. Philips-Roxane, Weesp, the Netherlands.

Received for publication, March 12, 1954.

Pyridoxine	80 mg.
Pyridoxal	80 mg.
Riboflavin	40 mg.
Nicotinic acid	40 mg.
Ca-d-pantothenate	40 mg.
Thiamine	40 mg.
Folic acid	20 mg.
p-Amino benzoic acid	20 mg.

The basal medium we used was similar to the medium of Skeggs and associates.[7] The stock cultures were incubated for eighteen hours at 37° C., and stored in a refrigerator at 4° C.

The serum samples were diluted eight to ten times, and 0.5, 1, 1.5, and 2 ml. of each dilution was pipetted into 120 by 12 mm. test tubes. Distilled water was added to make 2 ml., and after that, 2 ml. of double strength basal medium was poured into the tubes.

The tubes were autoclaved for three minutes at 120° C., and incubated for forty hours at 37° C. After incubation, the pH of the suspension in the tubes was measured with a Philips pH-meter.

Before starting the clinical determinations of the vitamin B_{12} activity, we tried different methods. The serum samples were treated in various ways.

1. Three milliliters of serum were diluted to 25 ml. The dilution was pipetted directly into the medium.

2. Three milliliters of serum plus 10 ml. of distilled water were heated for forty-five minutes at 100° C., cooled, and diluted under sterile conditions to 25 ml. The dilution was pipetted into the sterilized basal medium. We tried this method in order to compare it with the method of Ross.[8]

3. Three milliliters of serum plus 20 mg. of taka-diastase plus 15 ml. of buffer, pH 4.5, were allowed to stand for twenty hours at 37° C.; then steamed for fifteen minutes on a steam bath, and cooled. The pH was adjusted to 6.8 and the solution was diluted to 25 ml. The procedure followed next was similar to Method 1.

4. Three milliliters of serum plus 20 mg. of trypsin plus 15 ml. of buffer, pH 7.2, were allowed to stand for twenty hours at 37° C., then steamed for fifteen minutes on a steam bath, and cooled. The pH was adjusted to 6.8, and the solution diluted to 25 ml. Further procedure similar to Method 1.

5. Three milliliters of serum plus 20 mg. of papain plus 5 μg of KCN plus 15 ml. of buffer, pH 4.5, were allowed to stand for twenty hours at 37° C., then steamed for fifteen minutes on a steam bath and cooled. The pH was adjusted to 6.8 and the solution diluted to 25 ml. Further procedure was similar to Method 1.

A survey of the vitamin B_{12} values obtained by the previously mentioned methods is given in Table I. The first three samples are mixed samples from normal persons. The fourth sample is from a patient with multiple myeloma with an increase of the β globulin fraction.

TABLE I. VALUES FOR VITAMIN B_{12} ACTIVITY OBTAINED BY DIFFERENT METHODS (IN MμG PER ML. SERUM)

METHOD	SAMPLE A	B	C	D
1	1.042	0.600	0.625	0.250
2	0.966	0.450	0.475	0.038
3	0.250	0.350	0.300	0.025
4	0.240*	0.575*	0.475*	0.175*
5	0.117	0.100	0.175	0.025

*Sample A is determined with trypsine Novo, samples B, C, and D with trypsine B.D.H.

For the determination of free vitamin B_{12} activity, we applied the following method: To three milliliters of serum, 10 ml. of 96 per cent ethanol were added. The precipitate was filtered off and washed with 5 ml. of 70 per cent ethanol. The filtrate was evaporated at 30° C., and diluted with distilled water to 25 ml. Then 0.5, 1.0, 1.5, and 2 ml. of this suspension were pipetted into the basal medium.

TABLE II. VITAMIN B_{12} ACTIVITY OF SERUM SAMPLES OF NORMAL PERSONS FOR *Lactobacillus leichmannii* (MμG. PER MILLILITER)

PERSONS	FREE	TOTAL
1	0.100	0.31
2	0.125	0.33
3	<0.025	0.35
4	0.125	0.35
5	0.090	0.40
6	<0.025	0.40
7	0.125	0.40
8	<0.025	0.50
9	0.120	0.50
10	0.160	0.62
11	0.120	0.70
12	<0.025	1.05
13		0.33
14		0.34
15		0.40
16		0.41
17		0.42
18		0.42
19		0.425
20		0.44
21		0.45
22		0.47
23		0.50
24		0.50
25		0.52
26		0.52
27		0.56
28		0.60
29		0.63
30		0.67
31		0.70
32		0.71
33		0.74
34		0.76
35		0.78
36		0.80

TABLE III. VITAMIN B_{12} LEVEL IN SEVENTEEN PATIENTS WITH PERNICIOUS ANEMIA IN RELAPSE (MμG PER MILLILITER)

1	<0.005	<0.005
2	<0.010	0.010
3	<0.005	0.020
4	<0.025	<0.025
5	<0.025	0.025
6	<0.025	0.030
7	0.040	0.070
8	0.080	0.075
9	0.025	0.080
10	0.025	0.090
11	0.050	0.120
12	0.050	0.150
13		0.080
14		0.090
15		0.140
16		0.160
17		0.175

After sterilization, inoculation, and incubation, the pH of the suspensions in the tubes was measured. The values obtained by this method agreed with the following method, which is nearly similar to the method of Ross with Euglena.

Two milliliters of basal medium were autoclaved with 1.5, 1.0, 0.5, and 0 ml. of distilled water, respectively. After cooling, 0.5, 1.0, 1.5, and 2 ml. of a serum diluted under sterile precautions were added aseptically to the sterilized medium. After inoculation and incubation, the pH of the solutions in the tubes was measured.

With the aid of the alcohol precipitation method, as described previously, and Method 1, we have determined the free and total vitamin B_{12} activity in serum of normal persons and in serum of patients with pernicious anemia and other types of megaloblastic anemia. We preferred Method 1 because it gave the highest values and also because it was the simplest procedure.

Folic Acid Estimations in Blood.—The technique we used was essentially that of Teply and Elvehjem[9] with a modification by van Foreest.[10] We estimated the folic acid activity in whole blood, i.e., the folic acid activity of the plasma and the cells.

One should realize that the results are determined by the PGA and the CF content. The data have therefore no absolute value, but are used for comparing normal and pathologic samples.

RESULTS

In normal persons the values for total vitamin B_{12} varied from 0.3 to 1.05 Mμg per milliliter (See Table II). These values are comparable with those obtained by Ross[8] and Mollin,[11] with the *Euglena gracilis* technique. Nucleotides may stimulate the growth of *Lactobacillus leichmannii* in the absence of vitamin B_{12}.[12] These substances do not act as growth factors for the Euglena, which is a more specific test organism. In contrast to Girdwood,[13] Rosenthal and Sarett[14] and we could not detect any B_{12} activity after heating the serum for thirty minutes at 100° C. at pH 11 and pH 12, respectively. The values were often less than 0.001 Mμg per milliliter of serum. We feel, therefore, reasonably confident in stating that the *Lactobacillus leichmannii* assay in serum is specific for vitamin B_{12}.

In pernicious anemia in relapse, we found values varying from 0.005 to 0.175 Mμg per milliliter. (See Table III.) In other megaloblastic anemias reacting to vitamin B_{12} treatment, the vitamin B_{12} levels were also low.

In most patients refractory to vitamin B_{12}, the vitamin B_{12} level was normal; in two patients, however (one case of sprue and one case of megaloblastic anemia of pregnancy), the vitamin B_{12} level was abnormally high.

The values for the folic acid activity of whole blood varied in forty-three normal persons from 2.9 to 24.1 Mμg per milliliter, averaging 8.05 Mμg per milliliter. (See Table IV.) In four cases of sprue, and in one patient with megaloblastic anemia of pregnancy, the folic acid level was low. In these patients, vitamin B_{12} was inactive, while PGA administration was effective. In some patients reacting to vitamin B_{12} administration (nutritional megaloblastic anemia, megaloblastic anemia of pregnancy, megaloblastic anemia due to gastrocolic fistula), the folic acid activity was within the normal range. In sixteen cases of Addisonian pernicious anemia, normal values were found in about one-half of them; in the other patients, the values were low. (See Table V.)

The effect of an intramuscular injection of a large dose of vitamin B_{12} (80 μg, in one case 60 μg) on the blood folic acid level was studied in seven previously untreated cases of pernicious anemia. The immediate effect was a fall in the folic acid activity, in some cases within two hours after the injection. In all cases in which the study was continued for seventy-two hours or more, a subsequent rise occurred. (See Table VI.)

TABLE IV. FOLIC ACID ACTIVITY OF WHOLE BLOOD FOR *Streptococcus faecalis* IN NORMAL PERSONS (MμG PER MILLILITER)

PERSONS		PERSONS	
1	2.90	23	7.50
2	2.90	24	7.90
3	2.90	25	8.00
4	2.95	26	8.20
5	3.00	27	8.50
6	3.50	28	8.60
7	3.80	29	8.60
8	4.10	30	9.00
9	4.50	31	9.30
10	4.60	32	9.80
11	4.80	33	10.40
12	4.90	34	10.50
13	5.40	35	10.80
14	5.60	36	10.90
15	5.80	37	12.10
16	5.90	38	12.50
17	6.00	39	12.75
18	6.10	40	13.50
19	6.10	41	14.40
20	6.30	42	23.10
21	6.80	43	24.10
22	6.90		

TABLE V. FOLIC ACID VALUES IN SIXTEEN PATIENTS WITH PERNICIOUS ANEMIA IN RELAPSE (MμG PER MILLILITER)

PATIENTS		PATIENTS	
1	0.00	9	4.25
2	1.50	10	5.50
3	2.00	11	5.50
4	2.25	12	5.50
5	2.40	13	7.30
6	2.40	14	7.60
7	2.40	15	16.20
8	3.75	16	18.50

TABLE VI. FOLIC ACID ACTIVITY IN THE BLOOD OF PREVIOUSLY UNTREATED PERNICIOUS ANEMIA PATIENTS AT DIFFERENT INTERVALS AFTER THE INJECTION OF VITAMIN B_{12}

PATIENTS	VIT. B_{12} (μG)	0 HR.	2 HR.	6 HR.	17 HR.	24 HR.	48 HR.	72 HR.	5 DAYS	12 DAYS
1	60	5.50	4.00			2.90				
2	80	7.60	8.70				4.60			
3	80	2.25	0.00			1.08		2.69	4.87	
4	80	2.40		1.3	3.0	1.80	2.20			
5	80	6.25	6.38			1.18		18.88		
6	80	2.40	2.50	3.1		2.60		3.75	4.20	3.10
7	80		3.00	2.0		0.50	1.25	3.00	6.50	6.30

DISCUSSION

Megaloblastic anemias have been classified on the basis of their reaction to treatment into two categories. In the first group, of which Addisonian

pernicious anemia is the best-known example, vitamin B_{12} relieves all the manifestations of the deficiency: the anemia as well as the nervous system lesions are improved. The second group, of which the tropical macrocytic anemia studied by Wills[15] is the classical representative, proved to be refractory to treatment with vitamin B_{12} or purified liver extract. Wills observed a good response to yeast or crude liver extract. Her studies were the first demonstration of the activity of two dietary antimegaloblastic factors. A similar view was expressed by Davidson and Davis.[16]

In cases of the first group, we find a low level of vitamin B_{12}, as might be expected. This supports the opinion that in this type of case we are dealing with a vitamin B_{12} deficiency. (See Table VII.)

The values for vitamin B_{12} are normal or high in the B_{12}-refractory group. A vitamin B_{12} estimation therefore enables one to find out whether the patient needs vitamin B_{12} or PGA.

The values for the folic acid activity of the blood were lower than normal in the cases which proved refractory to vitamin B_{12}. The reaction to PGA or Leucovorin administration, as well as these laboratory estimations, indicates that the second group is due to folic acid deficiency.

In some of our patients who reacted to vitamin B_{12} (nutritional megaloblastic anemia, megaloblastic anemia of pregnancy, megaloblastic anemia due to gastrocolic fistula), the folic acid activity was within the normal range.

In our experience, megaloblastic anemia of pregnancy may react to treatment with vitamin B_{12}.[17] Sometimes, however, vitamin B_{12} is ineffective in which case PGA rapidly relieves the signs and symptoms. As might be expected from the effects of treatment, the results of folic acid and vitamin B_{12} assay are variable. One patient had a low vitamin B_{12} level with normal folic acid activity, another one, a low folic acid value with a high B_{12} level. The current opinion (Wintrobe,[18] Jukes,[19] Thompson and Ungley[20]), however, is that the folic acid is the deficient factor in this disease. Our experience confirms the view expressed by Watson and Castle,[21] that in megaloblastic anemia of pregnancy, one might deal either with a lack of extrinsic factor or with a deficiency of Wills' factor.

Sixteen cases of pernicious anemia were studied. Normal folic acid values were found in about one-half of these cases; in the other patients, the values were low. The reaction to treatment with vitamin B_{12} alone was adequate in all these patients. Additional folic acid administration was not required for producing a complete remission. Folic acid estimations are, therefore, less useful than vitamin B_{12} determinations for predicting the response to treatment in cases of megaloblastic anemia.

The problem of folic acid metabolism in pernicious anemia is complicated. The first theory on the interrelationship of vitamin B_{12} and folic acid was that proposed by Bethell and associates,[22-24] and Welch and Heinle and their associates.[25, 26] They supposed that vitamin B_{12} was essential for the liberation of PGA from the naturally occurring conjugate. According to this hypothesis,

TABLE VII. RESULTS OF FOLIC ACID AND VITAMIN B_{12} ESTIMATIONS IN DIFFERENT CONDITIONS

SEX AND AGE	DIAGNOSIS	RESPONSE TO TREATMENT—CLINICAL FEATURES	TIME	VIT. B_{12} FREE	VIT. B_{12} TOTAL	FOLIC ACID
1. ♀ 52	Nutritional megaloblastic anemia	Low-protein diet for nearly two years because of severe renal disease. Response to vit. B_{12}	Before treatment		0.175	16.40
			Two days after treatment			5.60
2. ♂ 50	Megaloblastic anemia due to gastric-colic fistula	Duodenal ulcer since 1925. Gastroenterostomy in 1937	Before treatment		0.130	10.30
3. ♂ 34	Nontropical sprue	Megaloblastic anemia refractory to vit. B_{12} reacting to PGA	Before treatment		0.950	
4. ♂ 36	Nontropical sprue	No anemia. Vitamin B_{12} ineffective. PGA improved general condition	Before treatment		1.900	2.30
5. ♂ 24	Nontropical sprue	Diabetes mellitus. Slight anemia. No megaloblasts or giant stabs in the bone marrow	Before treatment			1.70
6. ♂ 56	Nontropical sprue	Slight anemia. No megaloblasts or giant stabs in the bone marrow	Before treatment		0.370	0.00
7. ♀ 65	Nontropical sprue	Megaloblastic anemia. Vit. B_{12}, 15 per day, by injection, gave a reticulocyte crisis of 10.5%. The bone marrow remained megaloblastic. After administration of Leucovorin, however, the megaloblasts disappeared	Before treatment	0.075	0.240	2.00
			Before treatment	0.083	0.192	0.63
			Before treatment			0.25
			After 1 week on vit. B_{12}			0.75
			After 2 weeks on vit. B_{12}, before Leucovorin was started	0.120	0.580	1.00
				0.133	0.580	1.00
				0.200	0.660	1.00

8. ♀ 38	Nontropical sprue	No megaloblasts in bone marrow. Some clinical improvement after vit. B_{12} and PGA	Before treatment	0.040 0.040 0.040	0.375 0.400 0.380*	1.10
			After treatment with vit. B_{12} and PGA		0.420	6.10
9. ♀ 53	Tropical sprue	Treated with liver extract			0.460	4.50
10. ♀ 23	Megaloblastic anemia of pregnancy	Vit. B_{12} produced clinical and hematologic remission. Reticulocyte crisis 16.9%				8.90
11. ♀ 26	Megaloblastic anemia of pregnancy	Vit. B_{12} produced clinical and hematologic remission. Reticulocyte crisis 17.3%		0.040 0.040 0.040 0.040	0.040 0.040 0.040 0.040*	
12. ♀ 26	Megaloblastic anemia of pregnancy	Took low protein diet. "Spontaneous" improvement after admission			0.230 1.600	4.00 4.90
13. ♀ 26	Megaloblastic anemia of pregnancy	Three times 45 μg vit. B_{12} by injection was ineffective. PGA produced clinical and hematological remission. Reticulocyte crisis 25.6%	Before treatment 1 week after treatment with PGA		0.600	0.50
14. ♀ 48	Monocytic leukemia	The bone marrow contained megaloblasts as well as atypical monocytes		0.250	0.375	0.50 1.10 1.25

*The values were obtained by the Euglena gracilis method. We wish to thank Dr. D. L. Mollin and Dr. G. I. M. Ross.

PGA is the factor essential for the normal formations of blood cells. After this theory was withdrawn by its originator, Welch,[27] it was still supported by Davidson (See Girdwood[28]). An argument against this theory can be found in our normal folic acid assay results in nine out of sixteen cases of Addisonian pernicious anemia in relapse.

It has also been suggested that in pernicious anemia the conversion of PGA into CF is disturbed.[18] As it is necessary to give CF in the same dose as PGA in pernicious anemia patients, if one wants to obtain a maximal hemopoietic response, it seems unlikely that a retarded conversion of PGA into CF is the essential defect in patients with a vitamin B_{12} deficiency.[29]

On the basis of microbiologic data, the theory has been proposed that vitamin B_{12} and folic acid act as coenzymes at different stages in the biologic synthesis of nucleoproteins (Wright and associates,[12] Jukes and associates[30]). Our observations on the initial decrease of folic acid activity in pernicious anemia patients after the injection of vitamin B_{12} are in agreement with this theory. The rate of blood cell formation, which was slowed down by the disordered nucleic acid metabolism caused by lack of vitamin B_{12}, is accelerated by the administration of this factor. For the increased cell production in the bone marrow, more folic acid is required. This is in accordance with the observation of Vilter and associates,[4] who studied the folic acid excretion in a case of pernicious anemia which was treated with 10 mg. PGA daily. About 60 per cent of this amount was excreted in the urine. After the administration of liver extract, only 25 per cent was excreted. Later on, the folic acid excretion returned to the previous level. This also seemed to indicate that vitamin B_{12} increases the utilization of folic acid by the hemopoietic system. Another incidental observation deserves to be mentioned in this connection. In one case of megaloblastic anemia of pregnancy, the high level of vitamin B_{12} (1.6 μg per milliliter) dropped to 0.66 μg one week after the institution of treatment with PGA.

The folic acid activity is low in a number of cases of pernicious anemia in relapse. It seems, therefore, unlikely that the influence of vitamin B_{12} on folic acid metabolism can be explained by accepting an interrelationship only in the tissues utilizing these substances. Witts and his associates[31, 32] expressed the opinion that in Addisonian pernicious anemia, a folic acid deficiency might exist. This provides an explanation for the therapeutic effect of folic acid in this condition. Alternative explanations, however, are possible. We think that folic acid deficiency is not the cause of the low folic acid levels in the blood of uncomplicated pernicious anemia, because vitamin B_{12} therapy without additional folic acid may correct all the clinical manifestations. After vitamin B_{12} administration, the folic acid levels return rapidly to normal in pernicious anemia. In a case of sprue, however, with low values for both vitamin B_{12} and folic acid, vitamin B_{12} injection did not cause an increase of the folic acid values. The bone marrow remained megaloblastic and it was necessary to administer Leucovorin as well, in order to produce a complete remission. The

anemia in this case, which seemed to be caused by a double deficiency, is, therefore, markedly different from Addisonian pernicious anemia, in which vitamin B_{12} is the only factor required for a complete remission.

The low levels of folic acid and the secondary rise after vitamin B_{12} injection may also be explained as a manifestation of impaired absorption in Addisonian pernicious anemia. In an extensive study, Girdwood[33] did not find an impairment of folic acid absorption in this condition. It is, however, possible that the improvement of appetite by the vitamin B_{12} administration causes an increased folic acid intake with the food.

Another possible explanation of the effect of vitamin B_{12} is a mobilization of folic acid from the depots. Girdwood[34] studied the folic acid content of the liver in a number of patients dying from different diseases. Most of the folic acid was in the form of CF. In a previously untreated patient with pernicious anemia, who had received 100 μg of vitamin B_{12} the day before his death, the folic acid in the liver consisted mainly of PGA. Spray and Witts[35] found a diminished excretion of CF in the urine after a test dose of PGA in pernicious anemia in relapse. It seems possible that the low folic acid activity in the blood in untreated pernicious anemia as well as the increase after seventy-two hours may be explained by assuming that folic acid is mobilized from the depots in the liver in the form of CF and that the conversion of PGA into CF proceeds rapidly after the administration of vitamin B_{12}. Jukes[36] could not demonstrate an increased synthesis of CF in rats after vitamin B_{12} injection. It is, therefore, not clear whether the secondary increase of the folic acid activity in pernicious anemia patients is caused by a direct catalyzing effect of vitamin B_{12} on the conversion of PGA into CF. It is possibly a nonspecific result of an improvement of liver function. It is well known that metabolic changes (fatty degeneration) occur in the liver of untreated pernicious anemia patients. The liver is the main site of the conversion of PGA into CF.[37]

In conclusion, one may say that the available data on folic acid metabolism in Addisonian pernicious anemia suggests that: (a) vitamin B_{12} administration causes an increased utilization of folic acid in the bone marrow; (b) vitamin B_{12} has either a direct or an indirect influence on the storage of folic acid in the liver. The possibility, however, of an increased intake of folic acid after the institution of vitamin B_{12} therapy is not excluded.

It is generally believed that folic acid and vitamin B_{12} are related to nucleic acid synthesis. This opinion is based on microbiologic studies (Stokes,[38] Stokstad,[39] Wright and associates[12]). A disturbance of nucleic acid synthesis is essential in the pathogenesis of human megaloblastic anemia. This is indicated by the effectiveness of large doses of thymine (Spies and associates,[1-3] Petrides,[5] Vilter and colleagues[4]), uracil (Vilter and associates[4]), and thymidine (Hausmann[6]).

According to the pentose, two types of nucleic acid may be distinguished: desoxyribose nucleic acids (DNA) and ribose nucleic acids (RNA). DNA contains the pyrimidine bases thymine and cytosine, RNA, cytosine and uracil. Another essential difference is the localization of RNA and DNA in the cell.

RNA occurs in the cytoplasm and the nucleolus of the cell, while DNA is found in the nucleus only and is considered an essential constituent of the chromosomes. During cell division, the number of chromosomes is doubled; this process involves a twofold increase of the amount of DNA.[40, 41]

When lack of vitamin B_{12} or folic acid slows down DNA production, a disturbance becomes noticeable in the tissues characterized by intensive DNA production,[42, 43] in other words, the tissues with frequent mitoses. On histologic examination, the bone marrow, as well as the gastrointestinal tract, shows many cell divisions. Studies on the daily turnover of DNA phosphorus with P^{32} demonstrated that it is most rapid in these tissues.

While these considerations may provide us with an explanation for the megaloblastic anemia, the leukopenia, and the glossitis which we observe in cases of folic acid or vitamin B_{12} deficiency, it is necessary to discuss another important feature of vitamin B_{12} deficiency, i.e., the nervous system lesions. In the adult organism, the nerve cell does not show cell division and the DNA turnover is low. The RNA content, however, is high. The relation of RNA to protein synthesis has been demonstrated by Caspersson and associates.[44]

In the nerve cell the intensive protein synthesis is necessary in order to maintain the integrity of the axon (Bodian[45]). The total mass of the axon is estimated to be approximately one thousand times the cytoplasmic mass of the perikaryon in some neurons. A continuous centrifugal flow of cytoplasmic material along the axon was demonstrated by Weiss and Hiscoe.[46]

According to Stern and associates[47] vitamin B_{12} influences RNA synthesis. Folic acid, however, seems to act mainly on DNA production, not on RNA synthesis, at least in the experiments of Prusoff, Teply, and King[48]; Hodson[49] reported that in the absence of folic acid the growth of *Lactobacillus casei* can be maintained by adding a DNA preparation to the medium. RNA had no effect in these experiments.

We have previously pointed out that this may provide an explanation for the clinical observation that vitamin B_{12} deficiency may lead to both nervous system lesions and hematologic disorders; the lack of folic acid, however, causes anemia, while the nervous system remains intact.[50, 51] This concept finds support in the experimental data of Alexander and Backlar.[52]

If lack of vitamin B_{12} leads to a disturbance of the RNA-protein synthesis mechanism, it is not suprising that manifestations are found especially in those parts of the nervous system where this process is most intensive, i.e., in the cells with the longest axons. Long ago van der Scheer and Koek[53] pointed out that the characteristic location of the nervous system lesions in pernicious anemia is in the longest axons.

Wright and associates[12] suggested that folic acid is essential for the production of thymine. A further step is the conversion of thymine into thymidine; at this stage vitamin B_{12} is active as a coenzyme, according to these authors. (See Fig. 1.) Vilter and associates, who made extensive clinical observations on the use of uracil and thymine, came to a similar conclusion. They explain

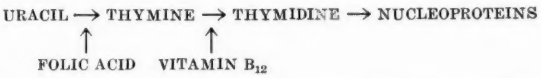

Fig. 1.—Interaction of folic acid and vitamin B_{12} according to Wright and associates (1948) and Vilter and associates (1950).

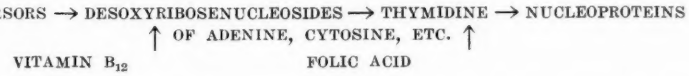

Fig. 2.—Interaction of folic acid and vitamin B_{12} according to Jukes, Broquist, and Stokstad (1950).

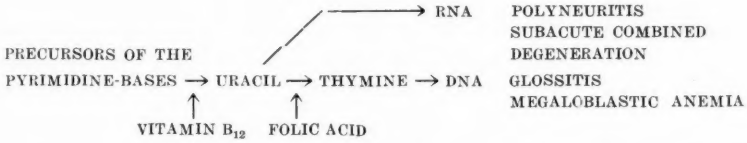

Fig. 3.—Interaction of folic acid and vitamin B_{12} according to the modified theory of Jukes, Broquist, and Stokstad.

the effect of folic acid in pernicious anemia by an increased synthesis of thymine. The production of thymidine proceeds at a normal rate in the pernicious anemia patient treated with folic acid, because the increased amount of the substrate available for the deficient coenzyme by mass action accelerates this step, which had been retarded. In the microbiologic experiment, however, nucleic acid constituents are thought to be growth-promoting in the absence of vitamin B_{12} or folic acid, because they are products of the processes catalyzed by these vitamins.

On the basis of more extensive microbiologic data, Jukes, Broquist, and Stokstad[30] suggested that folic acid is essential for the formation of thymidine from other desoxyribosides. The synthesis of these desoxyribosides is catalyzed by vitamin B_{12}. (See Fig. 2.)

From the clinical point of view, this theory has not received the attention which it deserves. We believe that it has definite advantages in explaining clinical observations. This theory indicates that vitamin B_{12} is necessary for the synthesis of different nucleosides, while folic acid is restricted in its activity to the conversion into thymidine, which is a constituent of DNA only. If it is permissible to modify this theory on the basis of observations from experiments with animals (Stern and associates, Alexander and Backlar), vitamin B_{12} is required not only for the synthesis of DNA, but also that of RNA. If we are not concerned with the question of whether or not the pyrimidine ring is closed before or after junction to the pentose, we can use the modified theory of Jukes, Broquist, and Stokstad, given in Fig. 3, to explain clinical data. Vitamin B_{12} is essential for the production of the pyrimidine ring of uracil and cytosine, while folic acid is thought to be responsible for the formation of thymine (5-methyluracil) from uracil.

If one looks at this diagram, it becomes clear why, in cases of folic acid deficiency, vitamin B_{12} is ineffective. In vitamin B_{12} deficiency, folic acid may correct the hematologic manifestations, because a large part of the limited

supply of pyrimidine bases is used for thymine formation. The amount of pyrimidine bases available for RNA synthesis will decrease even more, which may lead to a more rapid, possibly explosive, development of nervous system lesions.[54] As long as sufficient vitamin B_{12} is available for the process of pyrimidine synthesis, even large amounts of folic acid will not cause damage to the nervous system. The inability of folic acid to produce nervous system lesions in iron deficiency anemia or pernicious anemia patients given sufficient vitamin B_{12} has been adequately demonstrated by Chodos and Ross.[55] It is also easy to see why Vilter and associates[4] found both uracil and thymine to be effective in Addisonian pernicious anemia, while uracil had no effect on a patient with megaloblastic anemia of pregnancy, who reacted to treatment with thymine. In another pregnancy, the same patient again had anemia which was refractory to vitamin B_{12}. Folic acid proved to be active in this case.

While this diagram may give an adequate explanation of clinical empirical facts, we wish to stress that it should be regarded as theoretical speculation only.

This is a purely nutritional hypothesis. There is no need for the more complicated theory involving the interaction of toxic and nutritional factors proposed by Ungley,[56] in order to interpret the clinical observations about the effects of folic acid and vitamin B_{12}.

SUMMARY

Clinical experience has demonstrated that megaloblastic anemias can be divided into two groups, one category in which neurologic disorders may occur, reacting to treatment with vitamin B_{12}, and a second category reacting to folic acid, but refractory to treatment with vitamin B_{12}. The authors have compared the vitamin B_{12} levels in the serum of these groups with the aid of *Lactobacillus leichmannii* A.T.C.C. Strain 313. In the first group, of which Addisonian pernicious anemia is the best-known representative, the vitamin B_{12} content of the serum is low. Of the second group, the tropical anemia described by Wills is the classical example. In this group, the vitamin B_{12} values are either normal or higher than normal, and the folic acid activity of the blood for *Streptococcus faecalis* is lower than normal, as might be expected.

In pernicious anemia, the results of folic acid assay were variable. In nearly one-half of our cases, the values were low. The effect of an injection of vitamin B_{12} on the folic acid activity in pernicious anemia was also studied. A rapid fall occurred, sometimes within two hours. In those cases in which the study was continued for seventy-two hours or more, a subsequent rise was found.

On the basis of these data it was argued that vitamin B_{12} and folic acid interact not only in the tissues as coenzymes at different stages of nucleic acid synthesis, but that vitamin B_{12} also influences, in some way, either the storage or intake of folic acid.

Finally the role of vitamin B_{12} and folic acid in the synthesis of the pyrimidine bases in the nucleic acids is correlated with the clinical empirical data on the effects of these factors.

REFERENCES

1. Spies, T. D., Frommeyer, W. B., Vilter, C. F., and English, A.: Antianemic Properties of Thymine, Blood **1**: 185, 1946.
2. Spies, T. D., and Stone, R. E.: Liver Extract, Folic Acid and Thymine in Pernicious Anaemia and Subacute Combined Degeneration, Lancet **1**: 174, 1946.
3. Frommeyer, W. B., Spies, T. D., Vilter, C. F., and English, A.: Further Observations on the Antianemic Properties of 5-methyl-uracil, J. LAB. & CLIN. MED. **31**: 643, 1946.
4. Vilter, R. W., Horrigan, D., Mueller, J. F., Jarrold, T., Vilter, C. F., Hawkins, V., and Seaman, A.: Studies on the Relationships of Vitamin B_{12}, Folic Acid, Thymine, Uracil and Methyl Group Donors in Persons With Pernicious Anemia and Related Megaloblastic Anemias, Blood **5**: 695, 1950.
5. Petrides, P.: Die Perniziosabehandlung mit Thymin und Folsäure, Deutsch. Arch. Klin. Med. **194**: 661, 1949.
6. Hausmann, K.: Haemopoietic Effect of Thymidine in Pernicious Anaemia, Lancet **1**: 329, 1951.
7. Skeggs, H. R., Nepple, N. M., Valentik, K. A., Huft, J. W., and Wright, L. D.: Observations on the Use of *Lactobacillus leichmannii* 4797 in the Microbial Assay of Vitamin B_{12}. J. Biol. Chem. **184**: 211, 1950.
8. Ross, G. I. M.: Vitmain B_{12} Assay in Body Fluids, Nature **166**: 270, 1950.
9. Teply, L. J., and Elvehjen, C. A.: The Titrimetric Determination of "Lactobacillus Casei Factor" and "Folic Acid," J. Biol. Chem. **157**: 303, 1945.
10. van Foreest, D.: Over foliumzuurdeficientie bij varkens, Academic Thesis, Amsterdam, 1950.
11. Mollin, D. L., and Ross, G. I. M.: Paper read at the International Congress of Clinical Pathology, London, 1951.
12. Wright, L. D., Skeggs, H. R., and Huff, J. W.: The Ability of Thymidine to Replace Vitamin B_{12} as a Growth Factor for Certain Lactobacilli, J. Biol. Chem. **175**: 475, 1948.
13. Girdwood, R. H.: Some Aspects of the Metabolism of Antimegaloblastic Substances in Man, Blood **8**: 469, 1953.
14. Rosenthal, H. L., and Sarett, H. P.: The Determination of Vitamin B_{12} Activity in Human Serum, J. Biol. Chem. **199**: 433, 1952.
15. Wills, L., and Evans, B. D. F.: Tropical Macrocytic Anaemia: Its Relation to Pernicious Anemia, Lancet **2**: 416, 1938.
16. Davidson, L. S. P., and Davis, L. J.: Advances in Internal Medicine II, New York, 1947, Yearbook Publishers.
17. Nieweg, H. O.: Megaloblastic Anemia of Pregnancy, Correspondence, Lancet **2**: 491, 1952.
18. Wintrobe, M. M.: Clinical Hematology, ed. 3, Philadelphia, 1951, Lea & Febiger.
19. Jukes, T. H.: B-Vitamins for Blood Formation, Oxford, 1952, Blackwell.
20. Thompson, R. B., and Ungley, C. C.: Megaloblastic Anaemia of Pregnancy and Puerperium, Quart. J. Med. **20**: 187, 1951.
21. Watson, J., and Castle, W. B.: Nutritional Macrocytic Anemia Especially in Pregnancy. Response to a Substance Other Than That Effective in Pernicious Anemia, Am. J. M. Sc. **211**: 513, 1946.
22. Bethell, F. H., Swendseid, M. E., Bird, O. D., Meyers, M. C., Andrews, G. A., and Brown, R. A.: Observations on the Utilization of Folic Acid (Vitamin Bc) and Vitamin Bc Conjugate in Pernicious Anemia and Macrocytic Anemia Following Gastrectomy, Univ. Michigan M. Bull. **12**: 42, 1946.
23. Bethell, F. H., Meyers, M. C., Andrews, G. A., Swenseid, M. E., Bird, O. D., and Brown, R. A.: Metabolic Function of Pteroylglutamic Acid and Its Hexaglutamyl Conjugate. Hematologic and Urinary Excretion Studies on Patients With Macrocytic Anemia. J. LAB. & CLIN. MED. **32**: 3, 1947.
24. Swendseid, M. E., Bird, O. D., Brown, R. A., and Bethell, F. H.: 2. Urinary Excretion Studies on Normal Persons. Effect of a Conjugate Inhibitor, J. LAB. & CLIN. MED. **32**: 23, 1947.
25. Heinle, R. W., and Welch, A. D.: The Role of Conjugated and Free Forms of Folic Acid in the Control of Pernicious Anemia. I. Clinical Observations. Ann. New York Acad. Sc. **46**: 343, 1946.
26. Welch, A. D., Heinle, R. W., Nelson, E. M., and Nelson, H. V.: The Role of Conjugated and Free Forms of Folic Acid in the Control of Pernicious Anemia. II. Biochemical Aspects, Ann. New York Acad. Sc. **48**: 347, 1946.
27. Welch, A. D.: The Present Status of Pteroylglutamic Acid and of Other Hemopoietic Agents, Fed. Proc. **6**: 471, 1947.
28. Girdwood, R. H.: Vitamin B_{12} and Related Factors: A Clinical and Experimental Review, Edinburgh M. J. **57**: 72, 1950.

29. Cartwright, G. E., Wintrobe, M. M., Broquist, H. P., and Jukes, T. H.: Biological Activity of a Derivative of the Citrovorum Factor, Proc. Soc. Exper. Biol. & Med. **78:** 563, 1951.
30. Jukes, T. H., Broquist, H. P., and Stokstad, E. L. R.: Vitamin B_{12} and "Citrovorum Factor" in the Nutrition of *Lactobacillus Leichmannii* and Leuconostoc Citrovorum. Arch. Biochem. **26:** 157, 1950.
31. Witts, L. J.: The Pathogenesis of Megaloblastic Anaemias, Lancet **2:** 367, 1951.
32. Spray, G. H., Fourman, P., and Witts, L. J.: The Excretion of Small Doses of Folic Acid, Brit. M. J. **2:** 202, 1951.
33. Girdwood, R. H.: A Folic Acid Excretion Test in the Investigation of Intestinal Malabsorption, Lancet **2:** 53, 1953.
34. Girdwood, R. H.: The Interrelationship of Factors That Influence the Megaloblastic Anemias, Blood **7:** 77, 1952.
35. Spray, G. H., and Witts, L. J.: Conversion of Folic Acid to the "Citrovorum Factor" in Health and Pernicious Anaemia, Brit. M. J. **2:** 62, 1952.
36. Jukes, T. H.: Folic Acid and Vitamin B_{12} in the Physiology of Vertebrates, Fed. Proc. **12:** 633, 1953.
37. Nichol, C. A.: On the Metabolic Alteration of Pteroylglutamic Acid, Proc. Soc. Exper. Biol. & Med. **83:** 167, 1953.
38. Stokes, J. L.: Substitution of Thymine for "Folic Acid" in the Nutrition of Lactic Acid Bacteria, J. Bact. **48:** 201, 1944.
39. Stokstad, E. L. R.: Isolation of a Nucleotide Essential for the Growth of *Lactobacillus Casei*, J. Biol. Chem. **139:** 475, 1941.
40. Boivin, A., Vendrely, R., and Vendrely, C.: Biochimie de l'hérédité, Compt. rend. Acad. Sc. **226:** 1061, 1948.
41. Swift, H. H.: The Desoxypentose Nucleic Acid Content of Animal Nuclei, Anat. Rec. **105:** 17, 1949.
42. Hevesy, G., and Ottesen, J.: Rate of Formation of Nucleic Acid in the Organs of the Rat, Acta physiol. scandinav. **5:** 237, 1943.
43. Andreasen, E., and Ottesen, J.: Studies on Lymphocyte Production, Acta physiol. scandinav. **10:** 258, 1945.
44. Caspersson, T. O.: Cell Growth and Cell Function, New York, 1950, W. W. Norton.
45. Bodian, D.: Nucleic Acid in Nerve Cell Regeneration, Symp. Soc. Exper. Biol. **1:** 163, 1947.
46. Weiss, P., and Hiscoe, H. B.: Experiments on the Mechanism of Nerve Growth, J. Exper. Zoöl. **107:** 315, 1948.
47. Stern, J. R., Taylor, M. W., and Russel, W. C.: Relation of Vitamin B_{12} to Liver Basophilia, Proc. Soc. Exper. Biol. & Med. **70:** 551, 1949.
48. Prusoff, W. H., Teply, L. J., and King, C. G.: The Influence of Pteroylglutamic Acid on Nucleic Acid Synthesis in *Lactobacillus casei*, J. Biol. Chem. **176:** 1309, 1948.
49. Hodson, A. Z.: Compounds With "Folic Acid" Activity, Arch. Biochem. **21:** 330, 1949.
50. Nieweg, H. O., van Buchem, F. S. P., and Stenfert Kroese, W. F.: Verslagen Ned. Vereniging voor Haematologie, Nederl. Tijdschr. geneesk. **95:** 3540, 1951.
51. Nieweg, H. O., van Buchem, F. S. P., and Stenfert Kroese, W. F.: Vitamin B_{12} and Pteroylglutamic Acid in the Treatment of Megaloblastic Anemias, Acta med. scandinav. **142:** 45, 1952.
52. Alexander, W. F., and Backlar, B.: Nucleic Acid Changes in Rat Nerve Tissue After Parenteral Administration of Vitamin B_{12}, Proc. Soc. Exper. Biol. & Med. **78:** 181, 1951.
53. van der Scheer, W. M., and Koek, H. C.: Peripheral Nerve Lesions in Cases of Pernicious Anemia, Acta psychiat. et neurol. **13:** 61, 1938.
54. Ross, J. F., Belding, H., and Paegel, B. L.: The Development and Progression of Subacute Combined Degeneration of the Spinal Cord in Patients With Pernicious Anemia Treated With Synthetic Pteroylglutamic (Folic) Acid, Blood **3:** 68, 1948.
55. Chodos, R. B., and Ross, J. F.: Effects of Combined Folic Acid and Liver Extract Therapy, Blood **6:** 1213, 1951.
56. Ungley, C. C.: The Pathogenesis of Megaloblastic Anaemias and the Value of Vitamin B_{12}, Brit. J. Nutrition **6:** 299, 1952.

INCIDENCE OF HEMOGLOBIN C TRAIT IN 505 NORMAL NEGROES: A FAMILY WITH HOMOZYGOUS HEMOGLOBIN C AND SICKLE-CELL TRAIT UNION

ROSE G. SCHNEIDER, PH.D.
GALVESTON, TEX.

SINCE the demonstration by Pauling, Itano, Singer, and Wells[10] that sickling erythrocytes contain a type of hemoglobin electrophoretically different from the normal, several other genetically determined types of human hemoglobin have been distinguished (Itano[3]).

Itano and Neel,[4] studying the inheritance of sickle-cell anemia, encountered two families in which one or more children had sickling erythrocytes and a hemolytic anemia of a milder form than that usually seen in sickle-cell anemia. Erythrocytes of only one parent in each of these families exhibited sickling, contrary to the usual finding of sickle-cell trait in both parents of a child with sickle-cell anemia.

Electrophoretic analysis of the hemoglobin of the nonsickling parent in each of these families revealed the presence of a hitherto unrecognized type of hemoglobin, now called hemoglobin C.[23] This new type was found together with normal hemoglobin in erythrocytes of some subjects and together with sickling hemoglobin in others. It migrates as a more positive ion (in cacodylate buffer pH 6.5) than does sickling hemoglobin, which in turn migrates as a more positive ion than normal hemoglobin (Itano and Neel,[4] Pauling and associates[10]).

Kaplan, Zuelzer, and Neel[6] have made a clinical and hematologic study of subjects whose erythrocytes contain hemoglobin C. They found that those individuals whose erythrocytes contain hemoglobin C and sickling hemoglobin present a clinical condition resembling sickle-cell anemia, but of a more benign form. This syndrome is called sickle-cell-hemoglobin C disease (Neel, Kaplan, and Zuelzer[9]) and is the expression of heterozygosity for the sickling and C genes, whereas sickle-cell anemia is believed the expression of homozygosity for the sickling gene.

Subjects whose erythrocytes contain hemoglobin C and normal adult hemoglobin (the manifestation of heterozygosity for C and normal adult types of hemoglobin) are said to have hemoglobin C trait (Neel, Kaplan, and Zuelzer[9]). Kaplan, Zuelzer, and Neel[5] found no clinical abnormalities associated with this condition, and the only consistent hematologic abnormality was an increased incidence of target cells. They also reported a shortened survival time of hemoglobin C trait erythrocytes transfused into normal recipients, but could not substantiate this finding in subsequent experiments (Kaplan, Zuelzer, and Neel[5]).

From the Tissue Metabolism Research Laboratory, University of Texas Medical Branch, Galveston, Tex.
Aided by United States Public Health grant No. RG-3058 (C).
Received for publication, April 15, 1954.

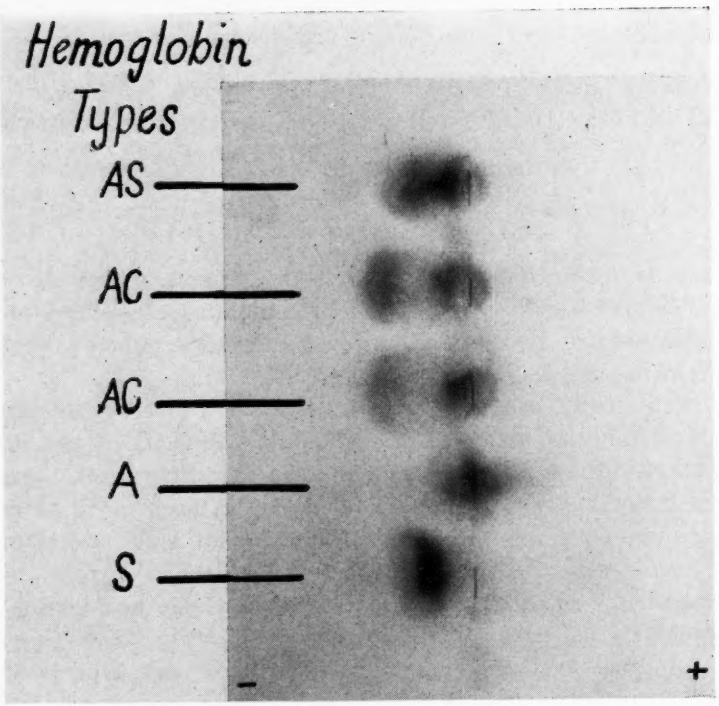

Fig. 1.—Differentiation of hemoglobin of normal adult (A), sickle-cell anemia (S), sickle-cell trait (AS), and C trait (AC) subjects by paper electrophoresis. Veronal buffer pH 8.7; 6½-hour run, 300 volts, 13-23 Ma.

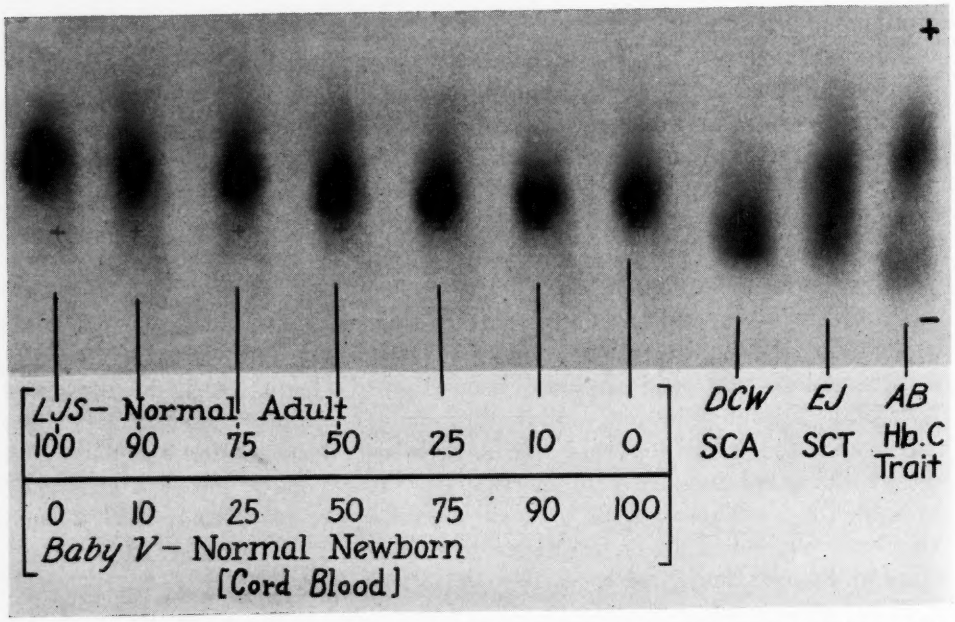

Fig. 2.—Differentiation of adult, fetal, C trait, and sickling (sickle-cell anemia, SCA; sickle-cell trait, SCT) hemoglobins in paper electrophoresis. Artificial mixtures of adult and fetal. Veronal buffer pH 8.6; 300 volts, 10 Ma., fifteen hours. DCW has 7.3 per cent fetal hemoglobin by alkali denaturation.

At the time this study was undertaken no reports of the incidence of hemoglobin C trait had appeared, but recently Smith and Conley,[20] using the method of filter paper electrophoresis between glass plates (Slater and Kunkel[19]), have found an incidence of 2.2 per cent hemoglobin C trait in 500 Negroes, while no cases of hemoglobin C trait could be found in 500 white individuals. Several instances of patients with erythrocytes containing C hemoglobin only (homozygous C) have recently been reported (Spaet, Alway, and Ward[22]; Ranney, Larson, and McCormack[12]; Watson[24]). All of these cases exhibited a more or less compensated form of hemolytic anemia. In Ashby survival time experiments (Spaet, Alway, and Ward[22]), the erythrocytes containing homozygous hemoglobin C had a shortened life span of about fifty-three days.

The application of the method of filter paper electrophoresis to differentiate electrophoretically distinct types of hemoglobin (Spaet[21]; Schneider[14]; Larson and Ranney[7]) has made possible large-scale electrophoretic analyses of hemoglobin samples. We have found that with this method, the following types of hemoglobin may be recognized: normal adult, fetal, sickling, and C. This is illustrated in Figs. 1 and 2.

A study of the incidence of hemoglobin C trait was undertaken by analysis of hemoglobin samples of normal Negro and white blood bank donors.* These donors are drawn from an area of several hundred miles. Hemoglobin samples of all patients with sickle-cell disease seen in this hospital during this time† and, wherever possible, hemoglobin samples of the families of these patients were also analyzed.

METHODS

Blood samples were obtained from consecutive blood bank donors for the study of the incidence of hemoglobin C trait. After the transfusion bottles were filled, about 5 to 10 ml. of blood from each donor were allowed to flow into sterile, evacuated, rubber-stoppered tubes containing 25 mg. of potassium oxalate. The blood from Negro donors was tested for the presence of sickling by the use of sodium metabisulfite (Daland and Castle[1]).

Preparation of Hemoglobin Samples.—The plasma and leukocytes were drawn off from the oxalated blood and the red cells washed three or four times in 0.85 per cent NaCl. After the final washing, an equal volume of distilled water and 0.4 volume of toluene were added to the packed cells, which were then shaken for five minutes and allowed to remain in the refrigerator overnight. The tubes were centrifuged for one hour at 2° C. at about 16,000 r.p.m., the clear hemoglobin solutions drawn off, and the concentrations determined in the Beckman DU Spectrophotometer. The concentrated hemoglobin solutions were usually diluted to 3 to 5 per cent with buffer before use.

Paper Electrophoresis.—The apparatus used (Fig. 3) is similar to that previously described (Schneider[13]), but has been enlarged so that several papers holding up to twelve samples each may be run simultaneously.‡ In practice, eight to ten samples were usually analyzed together with one normal and one sickle-cell anemia hemoglobin sample as controls. All but a few samples have been run two or more times.

*We are indebted to Miss Jean Stubbins, technical director of the Blood Bank, John Sealy Hospital, and her staff, for cooperation in obtaining these samples.

†Clinical studies were under the direction of Dr. Wm. C. Levin, Director, Hematology Research Laboratory, University of Texas Medical Branch, Galveston, Texas.

‡We are indebted to Mr. Jack Neal, Associate Professor of Industrial Hygiene, Department of Preventative Medicine and Public Health, University of Texas Medical Branch, Galveston, Texas, for valuable suggestions in the design of the apparatus.

The apparatus is made entirely of plexiglas and contains two chambers, 11 by 27.5 by 8 cm. high and 7.5 cm. apart. Each chamber is divided in half lengthwise by a baffle, to form an electrode chamber and a paper chamber. The baffle is elevated slightly from the floor, permitting free current flow between electrode and paper chambers while minimizing convection currents and pH changes in the latter. Fitting into each end of the 7.5 cm. space between the chambers is a Y-shaped piece of plexiglas, in the center of which is a vertical bar grooved to support the middle of the paper. The presence of several grooves at different levels makes it possible to use several papers simultaneously. The arms of the Y hold plexiglas rods over which the paper hangs with the ends suspended in buffer. These rods are elevated slightly above the level of the center of the paper.

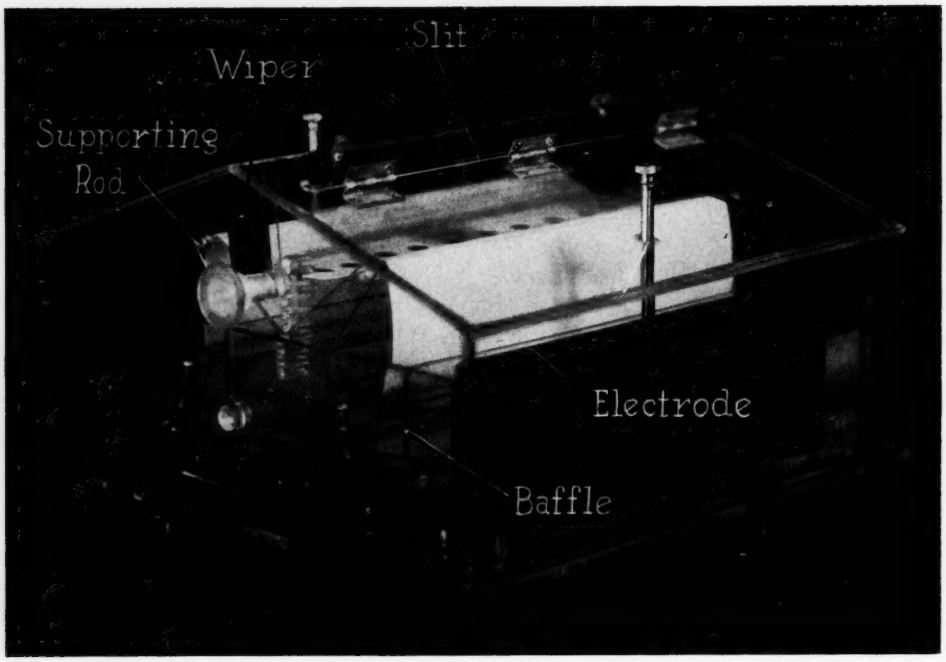

Fig. 3.—Paper electrophoresis apparatus.

The electrodes consist of flat sheets of stainless steel 26 by 9 cm. A stainless steel stem in the center of each electrode passes through a hole in the cover of the apparatus, and wires connected by screw nuts to this stem lead to the power source. The latter is a variable voltage regulated power supply capable of delivering 460 volts; with this voltage and 10,000 ohms resistance, using filter paper of 25 cm. width, fairly constant current readings of 13 Ma. are obtained. On one side of the tightly fitting cover is a hinged slit, through which pipettes may be passed so that evaporation is minimized during application of the hemoglobin drops. The cover becomes fogged during the course of a run; to improve visibility, a device similar to a windshield wiper is used, consisting of a rubber blade on the inside of the cover operated by a knob on the outside.

The apparatus is set on the level floor of a refrigerator at 5 to 10° C. Four liters of Veronal buffer of 0.05 ionic strength, pH 8.6, are introduced and the levels equalized by rubber tubing connecting the electrode chambers. This tubing is closed by two screw clamps so that there is no liquid junction between the electrode chambers to produce lateral current flow and therefore lateral migration of the hemoglobin. By reversing the wires from the anode and cathode leads at the end of each run, the same buffer may be used repeatedly, although small changes in ionic strength occur with evaporation of the water. Some analyses have been made with Veronal buffer pH 8.4 to 8.9.

Munktell No. 20 paper is cut in 25 by 34 cm. sheets. In the center, lines or crosses 2 cm. apart mark the location of the hemoglobin drops. The middle of the paper is fitted at each end into the plexiglas grooves. Lines 6 cm. from the center over the width of the paper mark the location of the plexiglas rods so that the paper may be hung over these evenly. The electrodes and cover are put into place and a thirty- to sixty-minute period of equilibration is allowed to elapse, during which time the paper becomes wet.

The hinged slit is now opened and 0.01 ml. of each hemoglobin sample is placed on the appropriate spot by means of pipettes introduced through the slit. The latter is then closed, current applied, and the doors of the refrigerator are closed on the wires leading to the power source.

At the conclusion of the run (usually overnight), the filter paper is removed and immediately smoothed down on the floor of a hot chromatographic oven to dry. The papers are stained by immersion in 95 per cent ethanol containing 0.1 Gm. per cent Brom Phenol Blue and 10 Gm. per cent $HgCl_2$. After about fifteen minutes in this stain, the papers are washed in running tap water until clear. More rapid migration without necessity of staining may be achieved by using 0.002 ml. of 10 to 15 per cent hemoglobin solutions. Recently, some experiments have been made with concentrated hemoglobin solutions between glass plates as described by Smith and Conley.[20] This method has been found rapid and convenient.

Tiselius Electrophoresis Determinations.—The Perkin-Elmer portable electrophoresis apparatus is used at a temperature of 1 to 2° C. Some analyses have been made with carboxyhemoglobin in cacodylate buffer pH 6.5, 0.1 μ, with a field strength of 2.37 or 5.16 volts per centimeter for six to eight hours, but other determinations have been made with oxyhemoglobin or carboxyhemoglobin in Veronal buffer pH 8.6, 0.1 μ, with a field strength of 11.37 volts per centimeter for two to three hours.* All hemoglobin samples were dialyzed overnight in the appropriate buffer; 0.5 to 1.5 per cent hemoglobin concentrations have been used.

Comments on Electrophoretic Methods.—In Veronal buffer, on the alkaline side of the isoelectric point, all types of hemoglobin migrate to the anode; the normal adult type has the greatest mobility (3.3 to 3.6 × 10^{-5} $cm.^2$ $volts^{-1}$ $sec.^{-1}$), followed by sickling hemoglobin (2.6 to 2.8 by 10^{-5} cm^2 $volts^{-1}$ $sec.^{-1}$), and hemoglobin C (2.1 to 2.4 × 10^{-5} $cm.^2$ $volts^{-1}$ $sec.^{-1}$), respectively.† These are also the relative mobilities of these types of hemoglobin in filter paper electrophoresis in Veronal buffer pH 8 to 9.0. Fetal hemoglobin could not be differentiated either from sickling or from adult hemoglobin in free electrophoresis in Veronal buffer pH 8.6, 0.1 μ.

One hemoglobin sample from a subject with repeatedly negative sickling tests resolved into two peaks in cacodylate buffer pH 6.5, similar to those found in sickle-cell trait. It was therefore thought to contain hemoglobin D (Itano[3]). In filter paper electrophoresis, this hemoglobin sample at first seemed to resemble that of sickle-cell trait (Schneider[13]). Since this was the expected result, it was not questioned at that time. On subsequent analysis, however, its migration was similar to that of the normal. Several analyses of hemoglobin samples from this patient in free electrophoresis in different buffers have given conflicting results, so that the original findings are in question. Another hemoglobin sample from a donor whose erythrocytes did not sickle in paper electrophoresis resembled the normal, but repeatedly lagged slightly behind. This hemoglobin sample resolved into two peaks of approximately equal area in free electrophoresis in cacodylate buffer pH 6.5. Additional blood from this donor was not available for further studies. At any rate, there appear to be hemoglobin samples which resolve into two peaks in free electrophoresis in cacodylate buffer and cannot be well differentiated from normal hemoglobin by the method of paper electrophoresis in Veronal buffer.

*We are indebted to John A. Cudd of the Tissue Metabolism Research Laboratory for the Tiselius electrophoresis analyses and the mobility figures.

†The mobility figures are for the descending patterns.

RESULTS

Hemoglobin samples from 60 white and 521 Negro donors have been analyzed. No sickling hemoglobin or hemoglobin C was found in any of the white donors. Of the 521 Negroes, 16 had AC hemoglobin and 60 had AS hemoglobin. All of the latter had positive sickling tests; of the 16 subjects with hemoglobin C trait, 15 were males, but since about 90 per cent of the donors were males, this finding is of little significance.

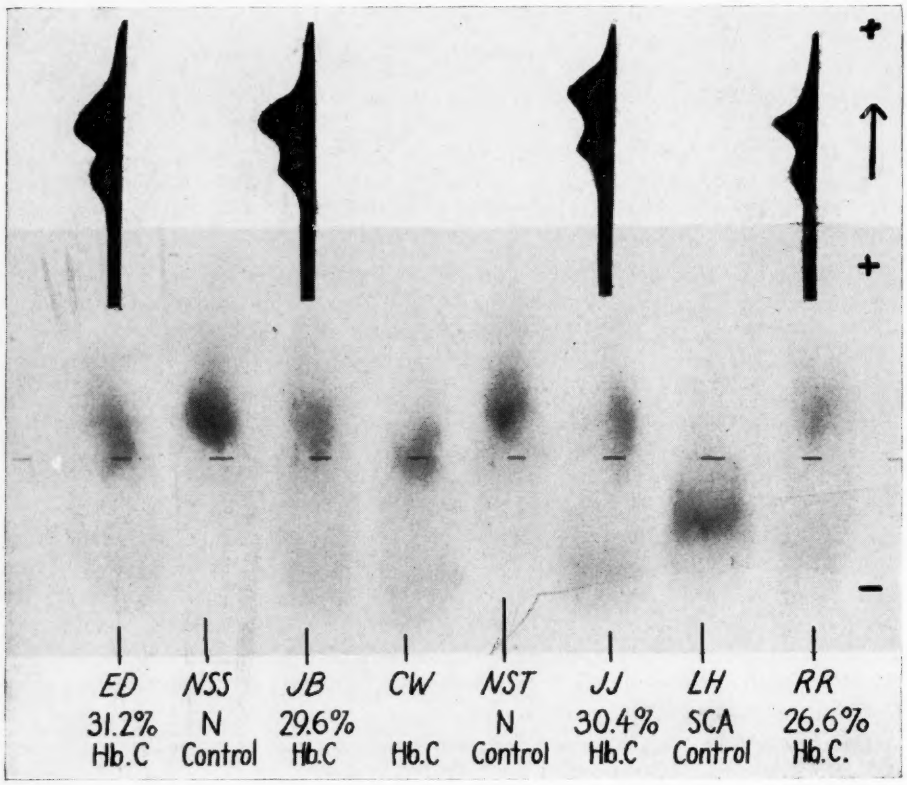

Fig. 4.—Electrophoretic patterns of hemoglobin C trait donors. Longsworth scanning diagrams of descending limbs, in Veronal buffer pH 8.6, 0.1 μ. Paper electrophoresis in Veronal buffer pH 8.4, 300 volts, 10 Ma.—sixteen hours.

Information about familial relationships was unavailable for most of the donors. Whenever several donors with the same family name and contributing to the same recipient appeared, however, only the first member of the group was included in the calculations. This resulted in the exclusion of 12 normal individuals, 3 with sickle-cell trait, and 1 with hemoglobin C trait, giving a final count of 505 donors of whom 57 had sickle-cell trait (11.3 per cent) and 15, hemoglobin C trait (3.0 per cent). With one exception, all the donors with hemoglobin C were widely separated in time or geographical location of their homes or both. The one exception consists of 2 donors with

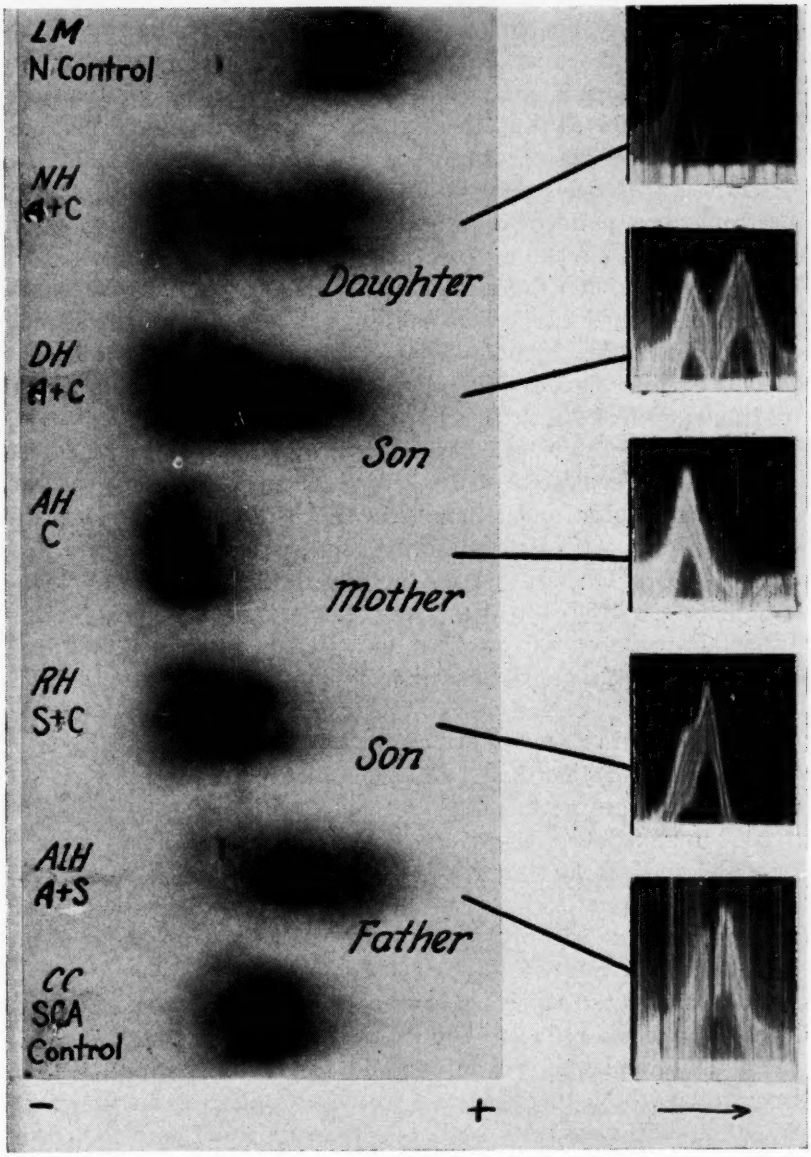

Fig. 5.—Electrophoretic patterns of hemoglobin of all members of Family H. Longsworth scanning diagrams of descending boundaries of hemoglobin samples in Veronal buffer pH 8.6; 0.1 μ and the corresponding paper electrophoresis patterns in Veronal buffer pH 8.9, 460 volts, 13 Ma., after six hours.

different family names, from the same city, whose blood was drawn on the same day for the same recipient. On questioning, no evidence of relationship between these two donors could be obtained.

Hemoglobin of several donors with negative sickling tests repeatedly lagged behind the normal. The reason for this is not clear; the possibilities present themselves that these samples may contain amounts of sickling hemo-

globin insufficient to cause sickling (Singer and Fisher[18]), or that they may contain types of hemoglobin that are not clearly detectable by present methods of filter paper electrophoresis.

Tiselius electrophoresis analyses in Veronal buffer pH 8.6, 0.1 μ, were made on hemoglobin samples of 5 of the sixteen donors with hemoglobin C trait. The percentages (average of ascending and descending patterns) of hemoglobin C in these 5 samples were 35, 34, 33, 33, and 28 per cent, respectively. Paper electrophoresis patterns of hemoglobin samples of these donors and corresponding Longsworth scanning diagrams of 4 of them are shown in Fig. 4. Two additional hemoglobin samples of hemoglobin C trait (in the family with homozygous C mentioned later) were subjected to free electrophoresis under the same conditions and were found to contain 34 and 38 per cent hemoglobin C, respectively. Similar figures for the percentage of hemoglobin C were obtained by Itano and Neel[4] in three cases of hemoglobin C trait.

During the study of Negro patients with sickle-cell disease, there were 5 whose hemoglobin consisted of a mixture of approximately equal amounts of sickle-cell hemoglobin and hemoglobin C. The clinical findings in these cases were consistent with those described by Kaplan, Zuelzer, and Neel[6] for sickle-cell-hemoglobin C disease. In free electrophoresis, hemoglobin of one of these patients resolved into two peaks of 46 per cent S and 54 per cent C, respectively. Both parents of another such patient were available for study. In paper electrophoresis, the father's hemoglobin resolved into A and S types while the mother's was entirely C. The latter finding was confirmed by free electrophoresis, both in cacodylate buffer pH 6.5 and Veronal buffer pH 8.6. Hematologically, the father had an uncomplicated sickle-cell trait; but the mother had a moderate normochromic anemia with 3,070,000 red blood cells and hemoglobin of 9.8 Gm. per cent. Negative sickling, a moderate number of target cells, and marked erythroid stimulation of the bone marrow were also noted. Alkali denaturation values for fetal hemoglobin (Singer, Chernoff, and Singer[16]) were within normal limits (0.8 per cent), as were those for the hemoglobin of the son with sickle-cell-hemoglobin C disease (0.9 per cent). The survival time of the mother's erythrocytes as determined by the Ashby technique was markedly reduced (about thirty days).

Hemoglobin samples of the father and the three children of this union were also analyzed both in paper and free electrophoresis in Veronal buffer. The hemoglobin of the son who initiated the study was found to be a mixture of S and C, while that of the two other children was C and A. Electrophoretic patterns of hemoglobin samples of all the members of this family is shown in

TABLE I. PERCENTAGE OF HEMOGLOBIN TYPES IN EACH MEMBER OF FAMILY H*

SUBJECT	RELATIONSHIP	A HEMOGLOBIN	S HEMOGLOBIN	C HEMOGLOBIN
Al H	Father	70.5	29.6	----
An H	Mother	----	----	100.0
R H	Son	----	62.6	37.4
N H	Daughter	62.6	----	37.5
D H	Son	65.9	----	34.2

*Averages of ascending and descending patterns in free electrophoresis in Veronal buffer pH 8.6, 0.1 μ.

Fig. 5. An account of the clinical and hematologic findings in this family has been reported (Levin, Schneider, Cudd, and Johnson[8]) and will be published in detail elsewhere.

The percentile distribution of the types of hemoglobin is summarized in Table I. The most noteworthy feature of this table is that although the father has only 30 per cent S hemoglobin, with 70 per cent A, the one child with sickling erythrocytes has about 60 per cent S and 40 per cent C.

DISCUSSION

The ease and economy of the method of filter paper electrophoresis make it readily applicable to analysis of large numbers of hemoglobin samples. A typical analysis is shown in Fig. 6. The most serious limitation of the method at present is the inability to obtain satisfactory results in acid buffers: there is indication that some types of hemoglobin may resolve differently in cacodylate and Veronal buffers, but filter paper electrophoresis with cacodylate and other acid buffers has been unsatisfactory.

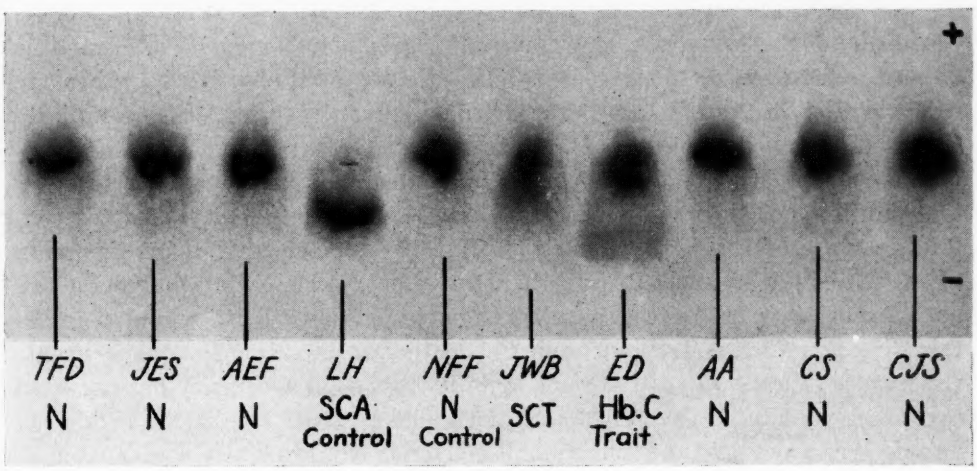

Fig. 6.—Typical paper electrophoresis analysis of hemoglobin samples. Veronal buffer 8.6; six and one-half hours, 300 volts, 10 to 20 Ma.

An attempt is being made to study the families of the hemoglobin C trait donors in order to obtain genetic data and, if possible, to find other cases of homozygous hemoglobin C.* To date, hemoglobin samples of both parents of one such donor have been examined and found to consist of normal adult hemoglobin (A) and hemoglobin C trait (AC), respectively. Hemoglobin samples of one parent of each of two other donors have also been found to consist of A and AC, respectively. In our family with homozygous C and sickle-cell trait (AS) union, two children had hemoglobin C trait (AC) and one, sickle-cell-hemoglobin C disease (SC). These results conform to expected Mendelian findings if the gene for hemoglobin C is a Mendelian dominant over the gene for normal hemoglobin and is unrelated to the gene for S hemoglobin.

*We are indebted to Miss Maureen Currin and the staff of the Texas State Department of Health for cooperation in obtaining these samples.

The method of quantitative inheritance of these types of hemoglobin is at present imperfectly understood. Our findings in seven individuals with hemoglobin C trait as well as those of Itano and Neel[4] in three such individuals indicate that these have a fairly constant amount of approximately 30 to 40 per cent hemoglobin C. In sickle-cell-hemoglobin C disease, the amount of C hemoglobin is about 40 to 50 per cent, both in our two cases and in those of Itano and Neel.[4] In the family in which the father has sickle-cell trait and the mother homozygous C, the father has about 30 per cent sickling hemoglobin combined with about 70 per cent normal; whereas the one son with sickling hemglobin has about 60 per cent of this type and about 40 per cent C, indicating that there may be a greater quantitative expression of the gene for S hemoglobin when associated with C than with A genes.

Another unsolved problem in this connection is the pathogenetic mechanism by which the presence of electrophoretically abnormal types of hemoglobin makes the cells susceptible to hemolysis. Our case of homozygous C, as well as those reported by others (Spaet, Alway, and Ward[22]; Ranney, Larson, and McCormack[12]; and Watson[24]), give clear evidence of hemolytic disease. The presence of such hemolytic disease and a shortened erythrocyte survival time in the absence of the morphologic anomaly of sickling indicate the need for re-examination of the commonly held hypothesis that, in sickle-cell anemia, it is the piling up of rigid, sickled cells in the small capillaries that initiates the hemolytic process. The possibility that erythrocytes containing types of hemoglobin different from the normal have a corresponding alteration of the stroma has never been ruled out. The finding that sickle-cell anemia erythrocytes have a peculiar immunologic specificity would point in this direction (Schneider and Levin[15]). Although in subsequent experiments (Schneider[14]) antibody of the same stability and high specificity has not been produced, we have repeatedly found that, under proper conditions, sickle-cell anemia erythrocytes (and some sickle-cell trait erythrocytes) are capable of producing rabbit antiserum which agglutinates many sickle-cell anemia erythrocytes, but not those of normal individuals nor of most subjects with sickle-cell trait.

The interesting finding of Ikin, Lehmann, and Mourant[2] that erythrocytes containing fetal hemoglobin possess a different immunologic specificity from that of normal adult erythrocytes again focuses attention on the question of the relationship of hemoglobin and erythrocyte stroma. In a recent discussion of this question, Ponder,[11] citing entirely different types of evidence, states: "The inference that the Hb. exists in the form of a complex with the lipoproteins of the cell surface is almost inescapable." At present experiments are still in progress to determine the precise condition under which specific antiserum against sickle-cell anemia erythrocytes may best be produced and to ascertain whether rabbit antiserum against erythrocytes containing homozygous hemoglobin C can also be formed.

SUMMARY AND CONCLUSIONS

The incidence of hemoglobin C trait among 505 Negro blood bank donors, as determined by the method of filter paper electrophoresis of the hemoglobin, was 3.0 per cent. The incidence of sickle-cell trait among these was 11.3 per cent.

Hemoglobin samples of seven individuals with hemoglobin C trait have been analyzed by free electrophoresis. The amount of hemoglobin C in these ranged from 28 to 38 per cent. Hemoglobin of two patients with sickle-cell-hemoglobin C disease have also been analyzed in free electrophoresis. In these, the amount of hemoglobin C was 37 and 55 per cent, respectively.

Electrophoretic data are presented on hemoglobin samples of one family with homozygous hemoglobin C and sickle-cell trait union. One of the children of this union has sickle-cell-hemoglobin C disease and the other two have hemoglobin C trait.

The question of the relationship of hemoglobin to the erythrocyte stroma is discussed.

We are indebted to Mrs. Leedia Greve Hanrahan of the Tissue Metabolism Research Laboratory, University of Texas Medical Branch, Galveston, Texas, for the area calculations of the Tiselius patterns and valuable assistance in preparation of the manuscript, and to Mrs. Mary Lou Fraser for technical assistance.

REFERENCES

1. Daland, G. A., and Castle, W. R.: A Simple and Rapid Method for Demonstrating Sickling of the RBC: The Use of Reducing Agents, J. LAB. & CLIN. MED. **33:** 1083-1088, 1948.
2. Ikin, E. W., Lehmann, H., and Mourant, A. E.: A Rabbit Serum Containing a Specific Agglutinin for the Red Cells of the Newborn, Brit. M. J. **2:** 602-608, 1953.
3. Itano, H. A.: Human Hemoglobin, Science **117:** 89-94, 1953.
4. Itano, H. A., and Neel, J. V.: A New Inherited Abnormality of Human Hemoglobin, Proc. Nat. Acad. Sc. **36:** 613-617, 1950.
5. Kaplan, E., Zuelzer, W. W., and Neel, J. V.: A New Inherited Abnormality of Hemoglobin and Its Interaction With Sickle Cell Hemoglobin, Blood **6:** 1240-1259, 1951.
6. Kaplan, E., Zuelzer, W. W., and Neel, J. V.: Further Studies on Hemoglobin C. II. The Hematologic Effects of Hemoglobin C Alone and in Combination With Sickle Cell Hemoglobin, Blood **8:** 735-746, 1953.
7. Larson, D. C., and Ranney, H. M.: Filter Paper Electrophoresis of Human Hemoglobin, J. Clin. Invest. **32:** 1070-1076, 1953.
8. Levin, W. C., Schneider, R. G., Cudd, J. A., and Johnson, J. E.: A Family With Homozygous Hemoglobin C and Sickle Cell Trait Union: A Clinical, Hematological, and Electrophoretic Study, Proc. Central Soc. for Clin. Res. Abstract No. 87, J. LAB. & CLIN. MED. **42:** 918, 1953.
9. Neel, J. V., Kaplan, E., and Zuelzer, W. W.: Further Studies of Hemoglobin C. I. A Description of Three Additional Families Segregating for Hemoglobin C and Sickle Cell Hemoglobin, Blood **8:** 724-734, 1953.
10. Pauling, L., Itano, H. A., Singer, S. J., and Wells, I. C.: Sickle Cell Anemia, A Molecular Disease, Science **110:** 543-548, 1949.
11. Ponder, Eric: Analytical Review: Present Concepts of the Structure of the Mammalian Red Cell, Blood **9:** 227-235, 1954.
12. Ranney, H. M., Larson, D. L., and McCormack, G. H.: Some Clinical, Biochemical, and Genetic Observations on Hemoglobin C, J. Clin. Invest. **32:** 1277-1284, 1953.
13. Schneider, R. G.: Paper Electrophoresis of Hemoglobin as a Practical Method of Differentiating Various Types of Sickle Cell Disease and of Hemoglobin "C" Trait, Texas Rep. Biol. & Med. **11:** 352-365, 1953.
14. Schneider, Rose G.: Unpublished experiments.

15. Schneider, R. G., and Levin, Wm. C.: Production of Specific Antisera Against Sickle Cell Anemia Erythrocytes; Antibody in Sicklemia Sera, Proc. Soc. Exper. Biol. & Med. **75:** 110-114, 1950.
16. Singer, K., Chernoff, A. I., and Singer, L.: Studies on Abnormal Hemoglobins. I. Their Demonstration in Sickle Cell Anemia and Other Hematologic Disorders by Means of Alkali Denaturation, Blood **6:** 413-428, 1951.
17. Singer, K., Chernoff, A. I., and Singer, L.: Studies on Abnormal Hemoglobins. II. Their Identification by Means of the Method of Fractional Denaturation, Blood **6:** 429-435, 1951.
18. Singer, K., and Fisher, B.: Studies on. Abnormal Hemoglobins: VI. Electrophoretic Demonstration of Type S (Sickle Cell) Hemoglobin in Erythrocytes Incapable of Showing the Sickle Cell Phenomenon, Blood **8:** 270-275, 1953.
19. Slater, R. J., and Kunkel, H. G.: Filter Paper Electrophoresis With Special Reference to Urinary Proteins, J. LAB. & CLIN. MED. **41:** 619-631, 1953.
20. Smith, E. W., and Conley, C. L.: Filter Paper Electrophoresis of Human Hemoglobins With Special Reference to the Incidence and Clinical Significance of Hemoglobin C, Bull. Johns Hopkins Hosp. **93:** 94-106, 1953.
21. Spaet, T. H.: Identification of Abnormal Hemoglobins by Means of Paper Electrophoresis, J. LAB. & CLIN. MED. **41:** 161-165, 1953.
22. Spaet, T. H., Alway, R. H., and Ward, G.: Homozygous Type "C" Hemoglobin, Pediatrics **12:** 483-490, 1953.
23. Statement Concerning a System of Nomenclature for the Varieties of Human Hemoglobin, Blood **8:** 386, 1953.
24. Watson, R. J.: The Hereditary Anemias, Bull. New York Acad. Sc. **30:** 106-121, 1954.

LABORATORY METHODS

THE CHEEK POUCH OF THE CHINESE HAMSTER *(Cricetulus griseus)* FOR CINEPHOTOMICROSCOPY OF BLOOD CIRCULATION AND TUMOR GROWTH

GEORGE P. FULTON, PH.D., BRENTON R. LUTZ, PH.D., DONALD I. PATT, PH.D., AND GEORGE YERGANIAN, PH.D.*
BOSTON, MASS.

WITH THE TECHNICAL ASSISTANCE OF KENNETH A. ARENDT, A.M., ROMA KAGAN, A.B., AND FREDERICK W. MAYNARD, A.B.

THE Chinese hamster, *Cricetulus griseus,*† has been inbred by Yerganian[1] for use in cytogenetic and tumor induction studies. In this species, the diploid chromosome number of 22,[2, 3] which is the lowest yet to be observed among the placental mammals, facilitates critical observation of mitosis and reduction division for the detection of aberrations arising from irradiation. The screening of induced tumors for possible chromosomal alterations, like those reported recently for rat ascites tumor cells,[4] is simplified in the Chinese hamster since each pair of chromosomes is morphologically different.

We have recently found that the cheek pouch of the Chinese hamster, like that of the golden species, *Mesocricetus auratus,*[5] may be everted and transillumined for visualization of living small blood vessels and for high-power motion picture photography. The purpose of this report is to direct attention to the usefulness of this new membrane preparation for peripheral vascular investigations and tumor growth studies.

METHODS

The cheek pouches are paired blind sacs lined with stratified squamous epithelium and located in the buccal wall, extending from the corner of the mouth posteriorly to the shoulder region. The experimental procedures involved in the preparation of the cheek pouch are similar to those described previously for the golden hamster.[5] The Chinese hamster is anesthetized by intraperitoneal injection of 1.0 mg. Nembutal per 12 grams of body weight. The anesthetized hamster is placed on a warm plastic operating dish containing a transilluminating block and adjacent pinning material (rubber or cork) mounted in a shallow moat, which may be kept filled with mammalian Ringer's solution at 37° C. Either pouch is everted by gentle traction with forceps, extended into the bath, spread over the transilluminating block, and pinned with "bank" pins to the adjacent rubber, forming a flat double-layered membrane (Fig. 1). The operating dish is transferred to an

From the Department of Biology, Boston University, Boston, Mass.
This investigation was supported by a contract with the Atomic Energy Commission, No. (30-1)-897.
Received for publication, Jan. 21, 1954.
*Public Health Service Research Fellow of the National Cancer Institute.
†Procurable from Mr. Victor Schwentker, Tumblebrook Farm, Brant Lake, N. Y.

electrically heated stage of a microscope equipped with a light-splitting prism, mounted between the objective and body tube, designed for simultaneous viewing and motion picture recording. As in the case of the golden hamster, microelectrodes and micropipettes may be applied by means of a micromanipulator, to the blood vessels in the rich vascular pattern of the cheek pouch. Kodachrome film, a 100 Watt zirconium lamp, and a "Cinekodak Special" motion picture camera mounted on an optical bench are used for cinephotomicroscopy.

After use, the unimpaired cheek pouch may be replaced. Repeated and periodic examinations of the same pouches are possible for long term chronic experimentation. Specific microscopic fields and particular blood vessels may be marked for reidentification by placement of a small, inert glass bead in the subepithelial connective tissue.

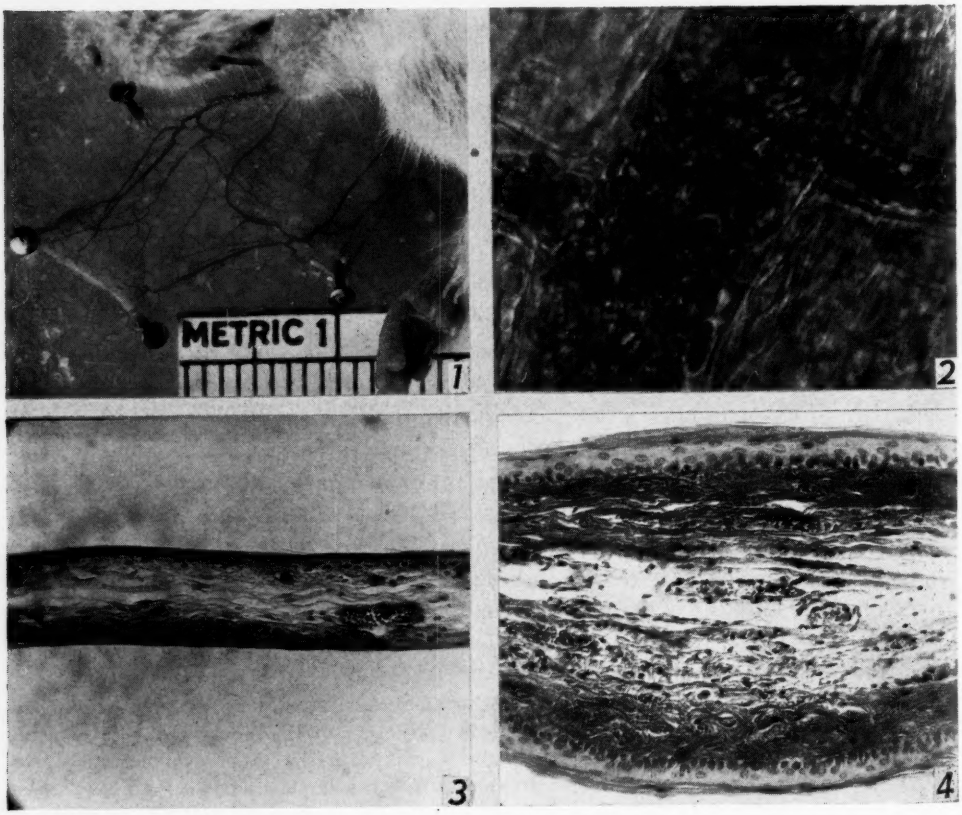

Fig. 1.—Everted cheek pouch of Chinese hamster, *Cricetulus griseus*, spread for transillumination of vascular pattern.

Fig. 2.—Cheek pouch venules showing circulating erythrocytes. (Magnification ×900; reduced ⅔.)

Fig. 3.—Section of everted cheek pouch of Chinese hamster. Thickness approximately 0.070 mm. (Magnification ×200; reduced ⅔.)

Fig. 4.—Section of everted cheek pouch of golden hamster, *Mesocricetus auratus*. Thickness approximately 0.500 mm. A large amount of loose connective tissue as compared with the Chinese hamster (Fig. 3). (Magnification ×200; reduced ⅔.)

For the purpose of transplanting tumors, the cheek pouch is everted and spread over a transilluminating block in the same manner as for circulatory studies. A small trocar may be used for implantation. The tip of the trocar is inserted through the upper layer of the double membrane and directed for advantageous positioning of the small tumor frag-

ment with respect to blood vessels. Finally, the pouch is unpinned and tucked back into the mouth cavity. The progress of the transplant may be followed with daily measurements if desired.

EXPERIMENTAL FINDINGS

The important assets of the Chinese hamster cheek pouch for vascular investigations are the thinness of the preparation, high degree of light transmission, and clarity of visualization. The rich vascular pattern is apparent in the everted pouch, spread as a membrane (Fig. 1). The comparative histology of the everted cheek pouches of the two species is shown in Figs. 3 and 4. High quality photography (Fig. 2) is possible through the double-layered membrane without the necessity of making a single-layered flap and removing the connective tissue, a procedure used to improve the quality of high power microscopy and especially photography in the golden hamster. The Chinese hamster has obvious advantages for investigation of the vascularity of tumor transplants because the growth of new blood vessels is easy to study in the thin pouch.

The cheek pouch of the Chinese hamster, like that of the golden hamster,[6, 7] also serves as a site for transplanting homologous and heterologous tumors. Yerganian has induced more than thirty methylcholanthrene fibrosarcomas in the Chinese hamster. Several have grown to a diameter of one-half inch within thirty days when transplanted into the cheek pouch. On the other hand, both spontaneous and methylcholanthrene-induced tumors (homologous) have been transplanted routinely into the cheek pouches of every "strain" of golden hamster with resultant growth.[6]

Possible disadvantages of the Chinese hamster are the delicacy of the pouch as compared with that of the golden hamster, and also the smaller size. Furthermore, the adult Chinese hamster weighs only approximately 35 grams as compared with 100 to 120 grams for the golden hamster. As with all small mammals, this imposes some problems in obtaining adequate blood samples and necessitates the use of micromethods. Because of the small body size, the depth of anesthesia must be carefully controlled.

DISCUSSION

The hamster cheek pouch has become an important addition to the list of useful living transparent membranes, which include tadpole tail, rabbit ear chamber, mouse skin chamber, wing membrane of bat, mesenteric spreads, and quartz rod transilluminated preparations. In the golden hamster, cinephotomicrographic investigations have been completed in our laboratory on white thromboembolism,[8] vascular fragility and anticoagulants,[9] irradiation effects on blood vessels and tissue mast cells, growth of transplanted malignant neoplasias, both homologous[6] and heterologous including human,[7] vascularization of tumor transplants, effects of *Trichinella spiralis*,[10] adrenal insufficiency and experimental hypercorticalism,[11] petechial formation, and effect of cortisone on healing of burns.

The value of the cheek pouch as a "living laboratory" should be enhanced by the use of the thin pouch in the Chinese hamster as a supplement to exten-

sive exploratory work with that of the golden species, especially in cases where high-power motion picture recording is required.

SUMMARY

The cheek pouch of the Chinese hamster, *Cricetulus griseus,* is described as a new living membrane preparation and compared with that of the golden hamster, *Mesocricetus auratus,* for use in cinephotomicroscopic investigations of the peripheral vascular system and for the study of tumor growth.

REFERENCES

1. Yerganian, G.: Cytogenetic Possibilities With the Chinese Hamster, *Cricetulus barabensis griseus,* Milne-Edw., Rec. Gen. Soc. Am. **21:** 82-83, 1952.
2. Matthey, R.: Les chromosomes des muridae, Experientia **7:** 340-341, 1951.
3. Moses, M. J., and Yerganian, G.: Desoxypentose Nucleic Acid (DNA) Content and Cytotaxonomy of Several Cricetinae, Rec. Gen. Soc. Am. **21:** 51-52, 1952.
4. Makino, S., and Kanô, K.: Cytological Studies of Tumors. IX. Characteristic Chromosome Individuality in Tumor Strain-Cells in Ascites Tumors of Rats, J. Nat. Cancer Inst. **13:** 1213-1235, 1953.
5. Fulton, G. P., Jackson, R. G., and Lutz, B. R.: Cinephotomicroscopy of Normal Blood Circulation in the Cheek Pouch of the Hamster, Science **105:** 361, 1947.
6. Lutz, B. R., Fulton, G. P., Patt, D. I., and Handler, A. H.: The Growth Rate of Tumor Transplants in the Cheek Pouch of the Hamster *(Mesocricetus auratus),* Cancer Res. **10:** 231-232, 1950.
7. Lemon, H. M., Lutz, B. R., Pope, R., Parsons, L., Handler, A. H., and Patt, D. L.: Survival and Growth of Human Tissues Transplanted to Hamster Cheek Pouch, Science **115:** 461-465, 1952.
8. Lutz, B. R., Fulton, G. P., and Akers, R. P.: White Thromboembolism in the Hamster Cheek Pouch After Trauma, Infection and Neoplasia, Circulation **3:** 339-351, 1951.
9. Fulton, G. P., Akers, R. P., and Lutz, B. R.: White Thrombo-embolism and Vascular Fragility in the Hamster Cheek Pouch After Anticoagulants, Blood **8:** 140-152, 1953.
10. Humes, A. G., and Akers, R. P.: Vascular Changes in the Cheek Pouch of the Golden Hamster During Infection With *Trichinella spiralis* Larvae, Anat. Rec. **114:** 103-113, 1952.
11. Wyman, L. C., Fulton, G. P., and Shulman, M. H.: Direct Observations on the Circulation in the Hamster Cheek Pouch in Adrenal Insufficiency and Experimental Hypercorticalism, Ann. N. Y. Acad. Sc. **56:** 643-658, 1953.

AN IMPROVED METHOD FOR MULTIPLE RAPID DETERMINATIONS OF ARTERIAL BLOOD pH

DUNCAN A. HOLADAY, M.D.
NEW YORK, N. Y.

RAPID fluctuations in the pH of arterial blood occur in response to alterations of effective pulmonary ventilation and attendant changes of CO_2 tension.[1, 2] In the course of a study of ventilation during anesthesia it became necessary to have means for monitoring the pH of blood frequently and conveniently. For this purpose it seemed desirable to have an instrument which would permit direct transfer of blood from the patient into a glass electrode, allow for determination of pH at body temperature, and be suitable for use in the presence of explosive gas mixtures in the operating room. No such apparatus is now commercially available. An instrument which meets these requirements and enables measurements to be made at two-minute intervals with an accuracy of 0.02 pH or better has been devised.

MATERIALS AND METHODS

Principles of Operation.—The apparatus consists of an electrostatically shielded air bath enclosing a condenser-type glass electrode (Figs. 1 and 2), which is connected by long, suitably shielded leads to a sensitive, null-point indicating potentiometer.* The air bath and electrode assembly are designed for safe use in the presence of explosive gas mixtures and may be placed beside the patient in the operating room. Small bore tubing conducts blood directly from an arterial cannula to the glass electrode. The potentiometer which contains a relatively high voltage electronic circuit is operated in an adjacent room or corridor outside the area where explosive anesthetic agents are used.

Shielding requirements are met by enclosing the electrode system entirely with copper screening, by using a doubly shielded coaxial cable for connection of the silver-silver chloride reference cell to the potentiometer, and by eliminating grounded circuit loops which could include the column of liquid contained in the glass electrode.

Since both the asymmetry potentials[3] of the several elements of the electrode system and the hydrogen ion concentration of blood[4] are altered by change of temperature, it proved necessary to maintain not only the air bath but also all liquids going through the electrode system at 37° C. ± 0.5°.

In compliance with the recommendations of the National Fire Protection Association,[5] heating elements are energized from a low-voltage isolation transformer, locking connectors are used throughout, and no open switches are employed. To provide for even heating of the air bath, a length and gauge of resistance wire were chosen, such that the current from a six-volt source generates heat at approximately one and one-half times the rate at which the air bath dissipates heat in a room at 22° C. At the same time, the temperature of the resistance wire is never elevated above 70° C. The latter condition

This work was supported by grants from the National Tuberculosis Association and The Squibb Institute for Medical Research.

From the Department of Anesthesiology, Columbia University, College of Physicians and Surgeons, and the Anesthesiology Service, The Presbyterian Hospital, New York 32, N. Y.

Received for publication, Feb. 10, 1954.

*Cambridge Instrument Co., Research model pH meter, No. 11261.

was imposed since no effort was made to make the air bath gas tight, and since 180° C. is the lowest temperature at which any mixture of anesthetic agent and oxygen will ignite spontaneously.[6] Final temperature regulation is obtained by means of a thermostat placed close to the heat source.

Preheating of all liquids entering the glass electrode is obtained in the following manner. A reservoir for a standard buffer solution is situated inside the air bath. Since 6 ml. of water are used for rinsing the glass electrode between determinations it was found expedient to heat it as it is consumed. Accordingly, before entering the air bath the water passes through a heating coil which has a volume capacity of 18 ml. and a heat output adequate to raise the temperature of 3 ml. of water 15° C. in one minute, but which cannot raise the temperature of the water above 37°. The small volume of blood withdrawn from the subject for each determination does not require temperature adjustment since the net loss of temperature at the electrode is less than 0.5° C.

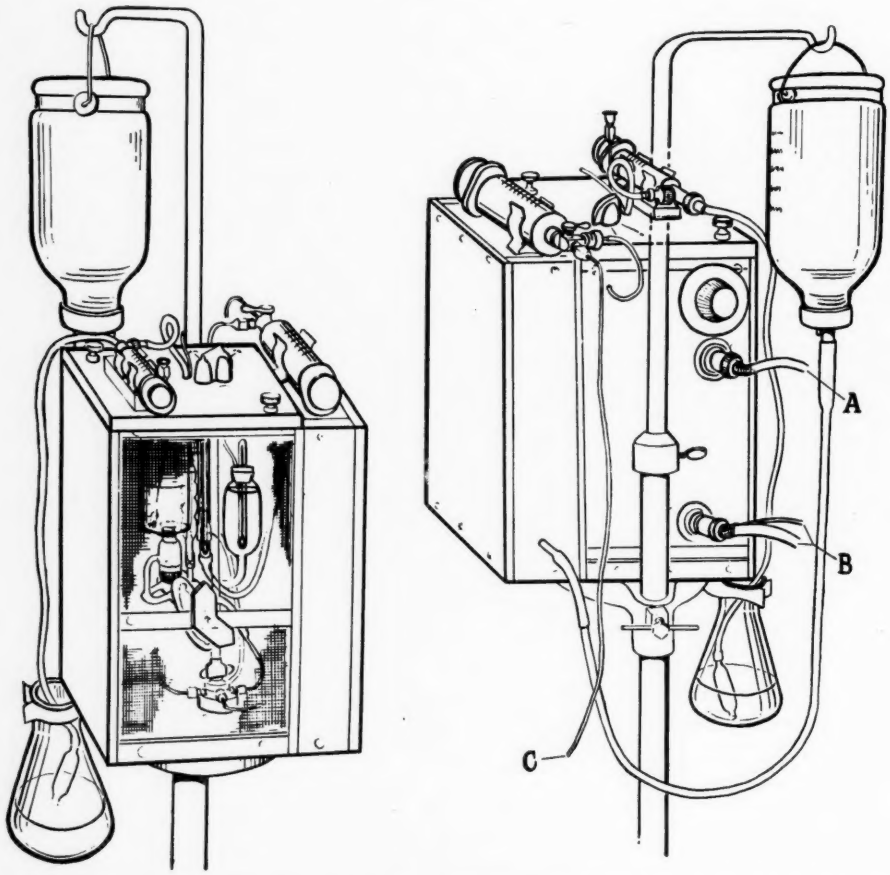

Fig. 1.—Shielded air bath containing modified electrode assembly. Left, front view showing position of glass electrode and external controls. Right, back view showing connections: *A*, to transformer; *B*, to potentiometer; *C*, to arterial cannula.

Construction.—The capitals in the following paragraphs refer to Fig. 2. The inner dimensions of the air bath are 4¾ inches by 5½ inches by 9⅛ inches. Two sides are made of ¼ inch Lucite, two sides are ¼ inch transite, and the top and bottom are ½ inch Lucite. The walls are supported by a framework of ⅛ by ½ inch angle brass set upon a base of ⅛ inch sheet brass. Stretched around the upright members of the frame, and

separated from the outer walls by a ⅛ inch air-space, is a continuous sheet of 16 mesh copper screening. Copper screening is fastened to the undersurface of the top of the box and makes firm contact with the brass frame when the top is bolted in place.

Studs screwed into the surface of each transite side support 4.75 feet (2.8 ohms) of No. 20 B & S gauge nichrome wire (A). A sheet of asbestos paper (B) insulates each heating element from the copper shielding. A sensitive bimetallic thermostat (C) projects into the box through one side and controls the current to the two heating elements which are connected in parallel.

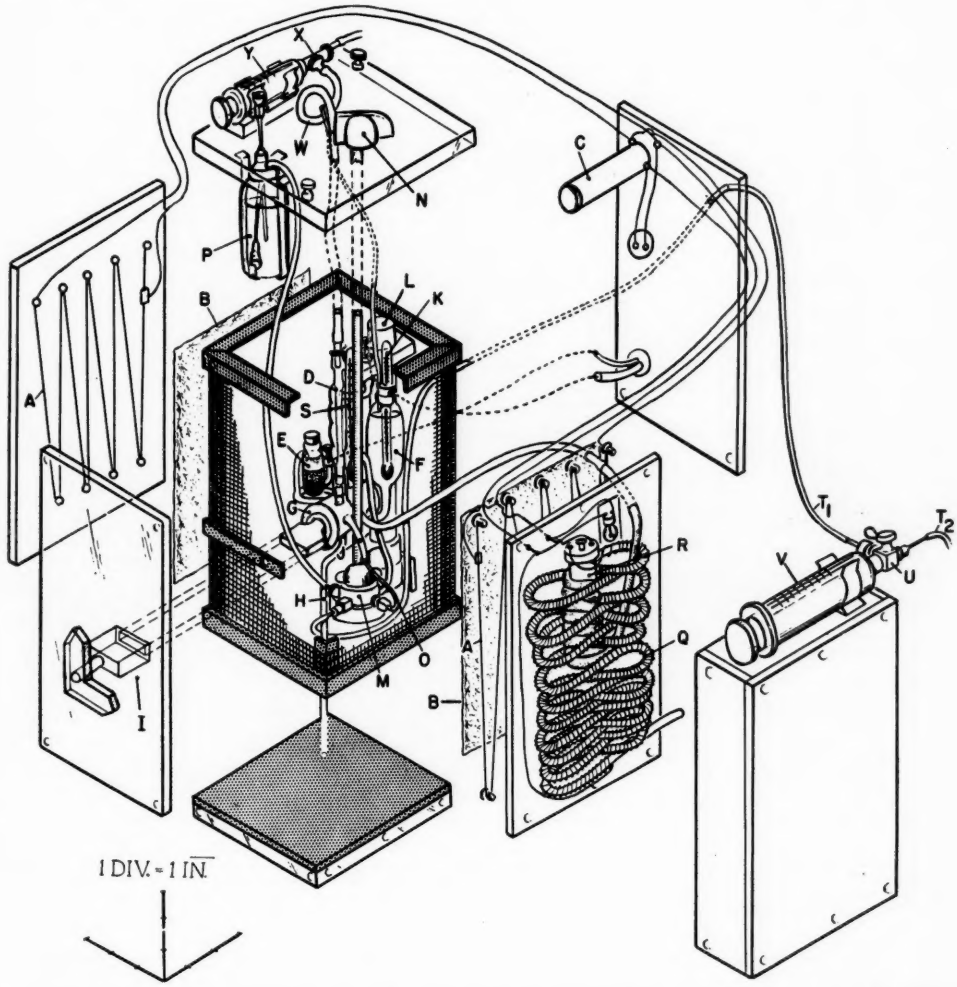

Fig. 2.—Exploded rectilinear diagram of air bath. *A*, Warming elements; *B*, asbestos paper; *C*, bimetallic thermostat; *D*, glass electrode and incorporated silver-silver chloride cell; *E*, calomel cell; *F*, KCl reservoir; *G*, 6-way stopcock; *H*, extra arm added to forward section of stopcock; *I*, 6-way stopcock adaptor; *J*, inlet tube of 6-way stopcock; *K*, thermometer; *L*, support rod for electrode assembly and 4-way stopcock; *M*, 4-way stopcock; *N*, 4-way stopcock adaptor; *O*, connecting tubing; *P*, buffer reservoir; *Q*, water warming coil; *R*, thermostat; *S*, thermometer; T_1, T_2, plastic tubing; *U*, 3-way stopcock; *V*, 20 ml. syringe; *W*, plastic tubing; *X*, valve; *Y*, 5 ml. syringe. Refer to text for details of construction.

The electrode assembly* is available commercially except for the glass stopcock, which has been modified to permit the salt bridge to be prepared without requiring move-

*Cambridge Instrument Co., No. 11284-1, SK-5697, and 13434.

ment of liquid toward the subject. The forward section of the stopcock is provided with four arms (see Fig. 3), through one of which (H), KCl drains by gravity. The stopcock is turned by means of a plastic adaptor (I), which projects through one side of the box. All liquids enter the glass electrode through one arm (J) of the six-way stopcock. The electrode assembly is supported by a ¾ inch rod of Lucite (L) which rests in a bracket fastened to the brass frame.

The Lucite rod also supports a four-way stopcock (M) near the inlet of the electrode assembly. The four-way stopcock is turned by means of a ¼ inch nylon rod adaptor (N) which projects through the top of the box. Polyethylene tubing (O) 3 inches long and 0.062 inch I. D. connects one arm of the four-way stopcock (M) to the inlet (J) of the glass electrode assembly. To reduce dead space, the tubing has been threaded through the inlet tube up to the stopcock and is cemented in place with a low melting point sealing wax. To the other three arms of the four-way stopcock, standard buffer, water, and blood are delivered via plastic tubing.

A fifty-milliliter bottle (P) containing the buffer is supported by light spring steel beneath the top of the box. The bottle can be filled through a perforation in the top of the box.

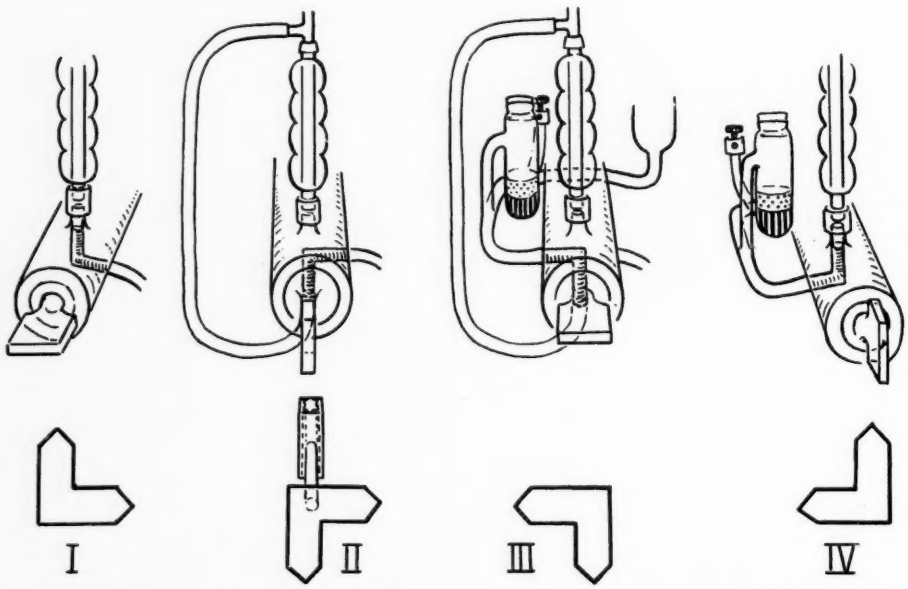

Fig. 3.—Positions of 6-way stopcock adaptor. *I*, first position connecting lumen of glass electrode to inlet tube; *II*, second position connecting inlet tube to lower outlet tube (H); *III*, third position connecting KCl reservoir and KCl bridge to lower outlet tube; *IV*, fourth position connecting glass electrode to calomel cell.

Distilled water from an external reservoir is conducted to the four-way stopcock by plastic tubing after passing through a warming coil (Q). The warming coil is enclosed in a plastic shell attached to one side of the air bath. It consists of a 9 foot length of cotton-wrapped copper tubing (0.125 inch I. D.) around which is coiled 20 feet (12 ohms) of No. 20 B & S gauge nichrome wire. The terminal 12 inches of copper tubing is stripped of insulation and is wrapped tightly around a bimetallic thermostat (R). The latter controls the current to the heating element. Current is drawn from the transformer which supplies the heating elements of the air bath. Interposed in the tubing between the warming coil and the four-way stopcock is a glass T-tube, one limb of which admits the bulb of a thermometer (S). The thermometer registers the temperature of the water entering the glass electrode.

Flexible plastic tubing (T_1 and T_2) 0.045 inch I.D., conducts blood from an intra-arterial needle through a perforation in one side of the box to the third inlet of the four-way stopcock. Interposed between T_1 and T_2 is a three-way stopcock (U) connected to a 20 ml. syringe (V). The syringe is clamped to the top of the box.

The upper opening of the glass electrode is connected by ⅛ inch I. D. flexible plastic tubing (W) to a one-way valve system (X). The latter is attached to a 5 ml. syringe (Y), which is clamped to the top of the box. The valve (X) permits flow from the glass electrode into the syringe and from the syringe through another piece of tubing into a waste flask suspended beneath the box. Thus fluid which has been selected by manipulation of the four-way stopcock is drawn into the electrode by the syringe (Y).

A doubly shielded lead connects the silver-silver chloride electrode to the potentiometer. The lead consists of twenty-five feet of low capacitance coaxial cable,* which has been covered by flexible copper webbing and wrapped with plastic insulation tape. The calomel cell is connected to the second input terminal of the potentiometer by an unshielded, insulated wire. The copper shielding of the box is connected to the inner shielding of the coaxial cable. Both cable shieldings and the case of the potentiometer are connected at a point near the potentiometer, from which point connection may be made to a water pipe or other suitable ground.

Operation.—To attain maximum stability, the electrode system must be protected from temperature fluctuations. The air bath is allowed to equilibrate at 37° C. for several hours before use. The thermometers (K & S) are read periodically and the thermostats (C & R) are readjusted as indicated to maintain temperatures at 37° C. ± 0.5°.

Asepsis of the blood inlet line (T_1 and T_2) up to the four-way stopcock (M) is maintained. The portion of this system within the box (T_1) is filled with 1:1,000 aqueous Zephiran Chloride solution when not in use. The three-way stopcock (U), syringe (V), and arterial needle are autoclaved before use. A supply of connecting catheters (T_2) are stored in Zephiran Chloride solution and rinsed with sterile saline solution before use. The syringe (V) is filled with sterile 0.9 per cent saline solution containing 0.1 mg. heparin per milliliter. This serves to rinse the tubing (T_1 and T_2) and prevent clot formation in the needle.

The six-way stopcock adaptor (I) is turned to the first position (see Fig. 3). The 4-way stopcock adaptor (N) is turned to admit buffer to the electrode. The glass electrode is filled with a standardized phosphate buffer by withdrawing the plunger of the 5 ml. syringe (Y).

To complete the salt bridge to the calomel cell, the six-way stopcock is turned to the third position momentarily (see Fig. 3) so that just the lumen of the stopcock plug is filled with saturated KCl solution. The six-way stopcock is turned to the fourth position. The potentiometer may then be read and adjusted to conform to the pH of the buffer. To prepare for the next measurement, the six-way stopcock is turned to the first position, the four-way stopcock is turned to admit distilled water, and the glass electrode is rinsed six times with 1 ml. portions of water. During each rinse, the tubing (W) may be compressed once or twice to produce more efficient washing of the glass electrode.

To introduce blood into the glass electrode, the four-way stopcock is opened to the blood line (T_1), the three-way stopcock (U) is turned to connect T_1 and T_2, and gentle suction is produced with the 5 ml. syringe (Y). Withdrawal of 1.5 to 2 ml. of blood from the subject is sufficient to fill the glass electrode with undiluted blood. The three-way stopcock (U) is then turned to connect the syringe (V) to the patient, and the tubing T_2 and needle are flushed with the saline and heparin solution. The three-way stopcock is turned to connect the syringe (V) to the tubing T_1 and the latter is partially filled with saline solution. The procedure hereafter is identical to that given in the preceding paragraph.

*Belden No. 8410.

An obstruction to the free flow of blood from the subject to the glass electrode is revealed by prompt collapse of the tubing (W) when traction is applied to the syringe (Y). The obstruction is usually in the needle itself and may be corrected by the insertion of a blunt stilet.

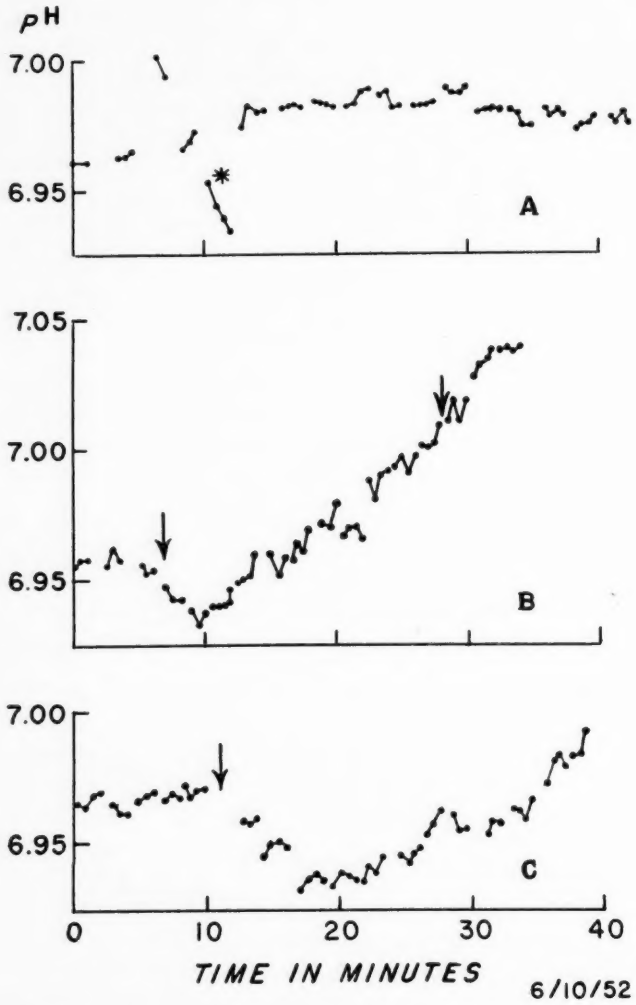

Fig. 4.—Serial pH determinations on phosphate buffer. Each solid circle represents an independent reading of the potentiometer; each short line joins those readings obtained on a single sample of buffer. Buffer was drawn from a warmed reservoir. The glass electrode was rinsed with six one-milliliter portions of water between each determination. *A*, The electrode was rinsed with water preheated to 37° C. Readings were taken on the fifth sample with the 6-way stopcock in the first position (KCl bridge not completed). *B*, Between the two arrows the electrode was rinsed with water at 27° C. *C*, After the arrow the electrode was rinsed with water at 26.8° C.

RESULTS

The stability of the electrode system and potentiometer was tested by making serial determinations on phosphate buffer drawn from the reservoir (P) within the air bath. During the course of a one-hour experiment (see Table I), the observed pH varied over a range of 0.007 pH. The standard

deviation from the mean was 0.002 pH. The average difference between successive readings was 0.0016 pH. The latter figure probably represents the average error incurred in reading the null point indicator of the potentiometer.

Fig. 4,*A* illustrates a similar experiment during which more frequent determinations were made. A gradual drift of 0.025 pH occurred. Again, however, the reading error, based on the difference between successive readings on individual samples, was negligible. It was commonly observed, as in this case, that the initial determinations of a series tend to be more variable than later determinations.

TABLE I. DETERMINATIONS OF PHOSPHATE BUFFER DRAWN FROM THE RESERVOIR AT INTERVALS OF FIVE MINUTES. THE ELECTRODE WAS NOT RINSED BETWEEN SAMPLES

READINGS	DIFFERENCE FROM MEAN	DIFFERENCE BETWEEN CONSECUTIVE READINGS
6.991	+0.001	
6.992	+0.002	0.001
6.992	+0.002	0.000
6.990	0.000	0.002
6.990	0.000	0.000
6.992	+0.002	0.002
6.991	+0.001	0.001
6.987	−0.003	0.004
6.988	−0.002	0.001
6.989	−0.001	0.001
6.990	0.000	0.001
6.989	−0.001	0.001
6.994	+0.004	0.005
Av. 6.990	S.D. = ±0.002	Av. 0.0016

$$\text{S. D.} = \sqrt{\frac{\Sigma\ (\text{x-mean})^2}{\text{n-1}}}$$

To test the effect of temperature gradients across the glass membrane upon stability, the electrode was rinsed with water of various temperatures between serial determinations of phosphate buffer. Figs. 4,*B* and 4,*C* illustrate two experiments in which water at room temperature was substituted for the 37° C. wash. The invariable result of cooling the glass electrode in this manner was an initial fall in the readings of 0.02-0.04 pH, followed by a rise beyond the original setting. Drifts exceeding 0.1 pH were observed. As is illustrated by Fig. 4,*B*, equilibration does not immediately follow resubstitution of water at 37° C. for rinsing.

The stability of response of the electrode system to blood was tested by performing alternate determinations on blood and on a phosphate buffer. Table II contains the crude data of one such experiment. Approximately 20 ml. of venous blood were drawn into an airtight syringe containing heparin and placed in a 37° C. water bath. Samples were withdrawn from the syringe into the glass electrode through small bore plastic tubing by the usual method. Each pH listed is the average of three readings obtained thirty seconds or more after withdrawal of the sample. The response to blood exhibited somewhat more variability than the response to buffer. Table III summarizes four of such experiments involving thirty-three determinations. The standard deviation of individual determinations about the corrected mean

TABLE II. ALTERNATE DETERMINATIONS ON PHOSPHATE BUFFER AND FRESHLY DRAWN, HEPARINIZED, VENOUS BLOOD

TIME (MIN.)	BUFFER pH	DEVIATION FROM MEAN	BLOOD pH	CORRECTED BLOOD pH	DEVIATION OF CORRECTED pH FROM MEAN	DEVIATION OF AVERAGE OF CONSECUTIVE PAIRS FROM MEAN
3	6.946	0.004				
11			7.286	7.299	+0.023	
14	6.938	0.004				
15			7.261	7.276	0.000	+0.012
17	6.941	0.001				
23			7.266	7.281	+0.005	+0.002
25	6.938	0.004				
27			7.252	7.268	-0.008	-0.002
30	6.939	0.003				
32			7.259	7.274	-0.002	-0.005
35	6.940	0.002				
37			7.259	7.269	-0.007	-0.004
40	6.950	0.008				
42			7.260	7.267	-0.009	-0.008
44	6.947	0.005				
Means	6.942	0.0039	7.263	7.276	= S.D. ±0.011	= S.D. ±0.007

Time is given in minutes from the moment the blood was collected in an airtight syringe and placed in a 37° C. water bath. The corrected blood readings were obtained by adding to the blood reading the difference between the average of the immediately preceding and succeeding buffer readings and 6.955 (the estimated true pH of the buffer at 37° C.).

pH was 0.018 pH; the standard deviation of the average of consecutive pairs of determinations was 0.012 pH. In three of the four experiments, there were one or two readings which varied more than .02 pH from the mean of the series. Deletion of the aberrant readings would contract considerably the previously mentioned confidence limits.

Variations in the readings of the order noted in the preceding paragraph occur most frequently during the first twenty minutes of operation. Bates and associates[7] have discussed several pertinent causative factors, among which is failure of the test solution to attain equilibrium in the double layer at the surface of the glass membrane after the electrode has been exposed to a buffer of different composition. Blood should remain in contact with the glass electrode for at least thirty seconds before the potentiometer is read. Measurements obtained within the first thirty seconds are frequently several hundredths of a pH unit lower than those obtained after the readings stabilize.

TABLE III. VARIATION AMONG pH DETERMINATIONS OF FRESHLY DRAWN WHOLE VENOUS BLOOD

EXP.	N	MEAN CORRECTED pH	INDIVIDUAL DETERMINATIONS S.D.	INDIVIDUAL DETERMINATIONS RANGE	AVERAGE OF CONSECUTIVE PAIRS S.D.	AVERAGE OF CONSECUTIVE PAIRS RANGE
1	7	7.276	0.011	+0.023 to -0.009	0.007	+0.012 to -0.008
2	10	7.252	0.030	+0.027 to -0.065	0.020	+0.025 to -0.036
3	7	7.272	0.012	+0.012 to -0.025	0.006	+0.004 to -0.012
4	9	7.254	0.004	+0.005 to -0.007	0.003	+0.005 to -0.002
Combined:	33		0.018*		0.012*	

*Combined S.D. $= \sqrt{\frac{n_1 \sigma_1^2 + n_2 \sigma_2^2 + \ldots}{(n_1 - 1) + (n_2 - 1) + \ldots}}$

See Table II and text for sample experiment.

Conversely, duplicate readings obtained as soon as possible after introduction of a phosphate or phthalate buffer into the electrode agree usually within the expected reading error.

The glass electrode becomes insensitive and unstable when allowed to remain dry for an extended period of time or when coated with a film of protein, grease, or silicone. The electrode should be kept clean with a neutral detergent such as Dreft or Tide, and should be filled with water when not in use.

The salt bridge of saturated KCl should be kept uncontaminated. A film of clotted fibrin across the salt bridge or a layer of blood protein at the surface of the calomel will cause instability. Air bubbles in contact with the glass membrane or in the salt bridge invariably produce gross errors.

DISCUSSION

Currently practiced methods[8, 9] for determination of hydrogen ion concentration of blood do not lend themselves easily to the task of following rapid changes over extended periods of time. Oil sealed syringes or tubes, containing anticoagulant and enzyme inhibitors, must be prepared in sufficient number beforehand. Because of the high diffusibility of carbon dioxide, care must be taken to insure anaerobic transfer of blood from patient to measuring apparatus.

The accuracy with which the pH of blood plasma may be determined is affected by temperature and enzymatic oxidation. Since blood continues to produce lactic acid and carbon dioxide after removal from the body, the pH will diminish appreciably with time unless the blood is chilled to 4° C. immediately upon withdrawal,[10] or unless an enzyme inhibitor such as sodium fluoride is added.[11] Errors due to oxidation are avoided most easily by analysis immediately after withdrawal.

The pH of blood changes linearly with changes of temperature between 4° and 40° C.[9, 10, 12] However, blood of different individuals differs in the magnitude of the change of pH with unit change of temperature. Graig and associates[9] have found a variation from +0.0103 to +0.0193 pH per degree Centigrade decrease in temperature in duplicate determinations of blood from twenty-seven individuals. Routine use of the mean coefficient as determined by them, 0.0149 pH per degree, for measurements at room temperature may result in errors as great as 0.08 pH. The data relating to this point were obtained presumably on venous blood within the normal range of pH. Dill, Daly, and Forbes[4] have shown that the temperature coefficient for plasma varies with its reaction. Blood having a more acid pH is changed less by change of temperature than is alkaline blood. For greater accuracy, it is necessary to determine the pH of blood at body temperature.

Accurate determinations of the pH of blood may be obtained only if maximum stability and sensitivity of the electrode and recording systems are maintained. Many things may cause instability. Several sources of error which were considered during the development of this method will be discussed.

Data have been presented which demonstrate the profound effect on short term stability resulting from temperature gradients across the glass electrode. With reference to the effect of temperature on the calomel cell, Wingfield and Acree[13] state that if readings precise to 0.01 or 0.02 pH are to be obtained, the cell system should be maintained at a constant temperature in an air or liquid bath for several hours before making measurements. The present study indicates that drifts as great as 0.1 pH may occur before reproducible buffer readings can be obtained when the instrument has not been permitted to equilibrate before use. Such drifts are characteristically slow and steady, however, and may be compensated for by frequent restandardization against known buffer.

The electrical resistance across the glass membrane is high, usually between 30 and 100 megohms. Consequently, a shunt across the two input leads, or between ground and the input lead from the silver-silver chloride cell (which goes to the grid of the detector tube) will diminish sensitivity. Moisture on the outer surfaces of the electrode or near the terminals may act as a significant shunt.

Another effect of the high impedence is to make the lead from the silver-silver chloride cell sensitive to small currents induced by local electrostatic and electromagnetic fields. This has been prevented in the present application by thorough shielding of this lead. A low capacitance coaxial cable with a single sheath of tinned copper webbing such as is used for microphone cable was found inadequate. A second sheathing proved necessary to eliminate instability from this source.

Sixty cycle interference from local power lines occasionally prevents precise reading of the null point indicator. This can be eliminated usually by correction of faulty grounding, elimination of multiple grounds, or by sealing some defect in the shielding.

Despite the many potential sources of error inherent to the glass electrode, meticulous technique during operation and maintenance of this instrument, and a thorough understanding of the problems associated with this type of apparatus will usually insure reliable determinations with a sensitivity adequate for most purposes.

CONCLUSIONS

A modified glass electrode assembly contained in a portable air bath which permits serial determinations of the pH of arterial blood at body temperature at two-minute intervals with an accuracy of 0.02 pH is described. Details of construction are presented. The apparatus is designed for safe use in areas where explosive gas mixtures are present. Some problems relevant to the determination of the pH of biologic fluids are discussed.

This method was developed for a specific problem which required frequent measurements of arterial blood over an extended period of time. It offers obvious advantages over other methods usually employed for routine pH determination of biologic fluids. The volume of fluid required for each determination is approximately two milliliters. The sample may be transferred

from the patient directly into the glass electrode through small bore tubing without exposure to air and without addition of anticoagulants or preservatives. The determination may be completed immediately at body temperature. The apparatus can be made ready for use again within one minute after completion of a determination.

ACKNOWLEDGMENTS

The author is grateful to Mr. H. F. Matthiessen of the Cambridge Instrument Co., for his generous counsel and to Mr. Robert Wade Speir for his patient assistance.

REFERENCES

1. Maier, H. C., Rich, G. W., and Eichen, S.: Clinical Significance of Respiratory Acidosis During Operations, Ann. Surg. **134:** 653-658, 1951.
2. Holaday, D. A., Ma, D., and Papper, E. M.: Dynamic Changes in Acid Base Balance During Anesthesia, Bull. N. Y. Acad. Med. **28:** 543, 1952.
3. Dole, Malcolm: The Glass Electrode, Chapter 9, New York, 1947, John Wiley & Sons.
4. Dill, D. B., Daly, C., and Forbes, W. H.: The pK′ of Serum and Red Cells, J. Biol. Chem. **117:** 569-579, 1937.
5. National Fire Protection Association Bulletin No. 56, Safe Practice for Hospital Operating Rooms, Boston, 1952.
6. Jones, G. W., Kennedy, R. E., and Thomas, G. J.: Explosion Hazards of Combustible Anesthetics, Bureau of Mines Tech. Paper 653, 1947.
7. Bates, R. G., Hamer, W. J., Manov, G. G., and Acree, S. F.: Provisional pH Values for Certain Standard Buffer Solutions. J. Res. Nat. Bur. of Standards, **29:** 183-190, 1942.
8. Hastings, A. B., and Sendroy, J., Jr.: Studies of Acidosis XX. The Colorimetric Determination of Blood pH at Body Temperature Without Buffer Standards, J. Biol. Chem. **61:** 695-710, 1924.
9. Graig, F. A., Lange, K., Oberman, J., and Carson, S.: A Simple, Accurate Method of Blood pH Determinations for Clinical Use, Arch. of Biochem. **38:** 357-364, 1952.
10. Wilson, R. H.: pH of Whole Arterial Blood. J. LAB. & CLIN. MED. **37:** 129-132, 1951.
11. Bueding, E., and Goldfarb, W. J.: The Effect of Sodium Fluoride and Sodium Iodoacetate on Glycolysis in Human Blood, J. Biol. Chem. **141:** 539-544, 1941.
12. Rosenthal, T. B.: The Effect of Temperature on the pH of Blood and Plasma in Vitro. J. Biol. Chem. **173:** 25-30, 1948.
13. Wingfield, B., and Acree, S. F.: Temperature and Hysteresis Errors in Calomel Half Cells. J. Res. Nat. Bur. Standards **19:** 163-175, 1937.

SIMULTANEOUS DETERMINATION OF SUCROSE AND INULIN IN BIOLOGIC FLUIDS

M. KENDALL YOUNG, JR., M.A.,* AND JOHN F. PRUDDEN, M.D., MED. SC. D.**
FORT SAM HOUSTON, TEX.

IN THE course of a study on methods for the measurement of extracellular fluid volumes in animals, an anthrone procedure for the determination of inulin which had many advantages over other methods was developed.[1] This procedure was adapted to the individual determination of sucrose and to its simultaneous determination with inulin.

Determinations of the volumes of distribution of inulin and sucrose on the same animal at different times[2] indicated that inulin had a smaller volume of distribution than sucrose. In order to confirm these findings, an accurate means of simultaneously measuring these substances of similar chemical properties was necessary. Keith and Power,[3] using a modified Shaffer-Hartman method, and Steinitz,[4] the resorcinol reaction, have reported simultaneous measurements of these materials by determinations before and after yeasting. Because of the high degree of reproducibility obtained with the anthrone technique, the adaptation of this method to the problem appeared desirable. The use of an invertase preparation also offered additional advantages over the procedures requiring yeasting.

MATERIALS AND METHODS

The anthrone reagent used in this study has been described previously.[1] This reagent was prepared by adding 250 ml. of an anthrone solution (4 Gm. of anthrone to 250 ml. 98 per cent sulfuric acid) to 750 ml. of a sulfuric acid solution (500 ml. 98 per cent sulfuric acid to 250 ml. water). Overheating must be avoided. An invertase preparation capable of completely inverting 10 per cent sucrose in one hour at room temperature was employed.†

Color Development.—Color development was carried out in the manner described for the determination of inulin.[1] Ten milliliters of anthrone reagent were added from an automatic acid burette to 2 ml. of sample containing 10 to 150 μg of sucrose and/or inulin. By shaking the test tube in cool water during addition of the color reagent, heat development was minimized. Tubes were shaken vigorously and placed in a constant temperature bath (75° C. ± 0.5°) for five minutes. The tubes were subsequently cooled (at least fifteen minutes) in tap water, and the color read at 630 mμ in the Coleman Junior Spectrophotometer.

Plasma.—Plasma proteins were precipitated by a modified Somogyi method using one part plasma, thirteen parts water, one part 10 per cent $ZnSO_4 \cdot 7\ H_2O$, and one part 0.525 N NaOH‡ When inulin was to be determined, the precipitate was shaken for twenty minutes in a mechanical shaker.[1] The protein precipitate was spun down and 4 ml. aliquots were placed in

From The Surgical Research Unit, Brooke Army Medical Center, Fort Sam Houston, Tex. Received for publication Feb. 23, 1954.

*Surgical Research Unit, Brooke Army Medical Center, Fort Sam Houston, Tex.

**Present address: Department of Surgery, Columbia-Presbyterian Medical Center, New York, N. Y.

†Available from Difco Laboratories, Inc.

‡Ninety-eight per cent recoveries of inulin were obtained with 0.525 N NaOH, whereas 93 per cent and 90 per cent were obtained with 0.5 N and 0.475 N, respectively. Concentrations of NaOH above 0.525 N gave recovery values greater than 100 per cent, indicating incomplete precipitation of protein.

tubes containing 1 ml. of 4 N NaOH, and the tubes were covered with a glass marble and heated for fifteen minutes at 100° C. The tubes were then cooled, and 2 ml. aliquots were taken for color development.

Urine.—Urine samples were diluted to approximate plasma concentrations, and determinations were carried out as described for plasma.

Invertase.—When sucrose and inulin were determined simultaneously, the optical density of inulin plus sucrose was determined in the manner described. The optical density* of inulin was then determined by incubating 1 ml. of original sample with a 1:10 dilution of invertase preparation and 5 ml. of water at 38° C. for sixty minutes. This solution was then diluted to 14 ml., the proteins were precipitated and the optical density was determined in the usual way. An invertase blank was prepared in the same way, utilizing 1 ml. of the invertase dilution and 1 ml. of a plasma or urine blank with appropriate corrections made for these blanks. The optical density of inulin was calculated in the following manner:

(1) $O.D.I._{Calc.} = O.D.I.S._{Inv.} - O.D._{Inv.}$

$O.D.I._{Calc.}$ = calculated optical density for inulin.

$O.D.I.S._{Inv.}$ = optical density of the sample incubated with invertase.

$O.D._{Inv.}$ = invertase blank.

The optical density of sucrose was calculated as:

(2) $O.D.S._{Calc.} = O.D.I.S. - O.D.I._{Calc.}$

$O.D.I.S.$ = the total optical density of sucrose and inulin.

$O.D.I._{Calc.}$ = the optical density of inulin as calculated above.

The concentration of inulin or sucrose was then calculated as:

(3) $$\text{mg. \% Unknown} = \frac{\text{O.D. Unknown}}{\text{O.D. Known}} \times \text{mg. \% Known.}$$

In order to test recoveries of inulin and/or sucrose, aliquots of standard were added to plasma or urine blanks, the optical density was determined, and per cent recoveries were calculated. To test the effectiveness of the invertase preparation, samples containing sucrose were incubated with various dilutions of the commercial invertase preparation and incubated for different intervals of time. The optical densities were then determined.

RESULTS

The effect of temperature on the development of the color intensity with sucrose was observed, and the results are given in Fig. 1. This figure shows that changes in color density exhibit a linear relationship with the concentration at any specified temperature. The temperature used in this study was 75° C.; maintained for five minutes. This offers the convenience of determining sucrose at the optimal temperature of inulin. As with inulin,[1] longer periods of heating only decrease color intensity.

The effective concentration of invertase and time of incubation required for complete inversion† of sucrose were tested. A 1:10 dilution of the Difco preparation proved capable of inverting all sucrose under the experimental conditions described previously, i.e., 1 ml. sample, 1 ml. invertase solution, 5 ml. water, incubated for sixty minutes at 38° C. In order to evaluate the effectiveness of the inversion of sucrose at high concentrations, samples containing as high as 120 mg. per cent sucrose (the maximum concentration expected in measurement of extracellular fluid volume) were tested and inversion was found to be 100 per cent.

*The color intensity of sucrose developed at 75° C. is somewhat less than that for the same equivalent of inulin so that individual optical densities must be determined.

†Inversion as used here is defined as complete disappearance of chromogen from sucrose after incubation with invertase.

The standard curve for sucrose is presented in Fig. 2. The linearity obtained demonstrates the reproducibility of this determination over a wide range of concentrations. Recoveries of sucrose following sodium hydroxide digestion were tested, and C.P. sucrose was found to be 97.8 ± 1.8 per cent

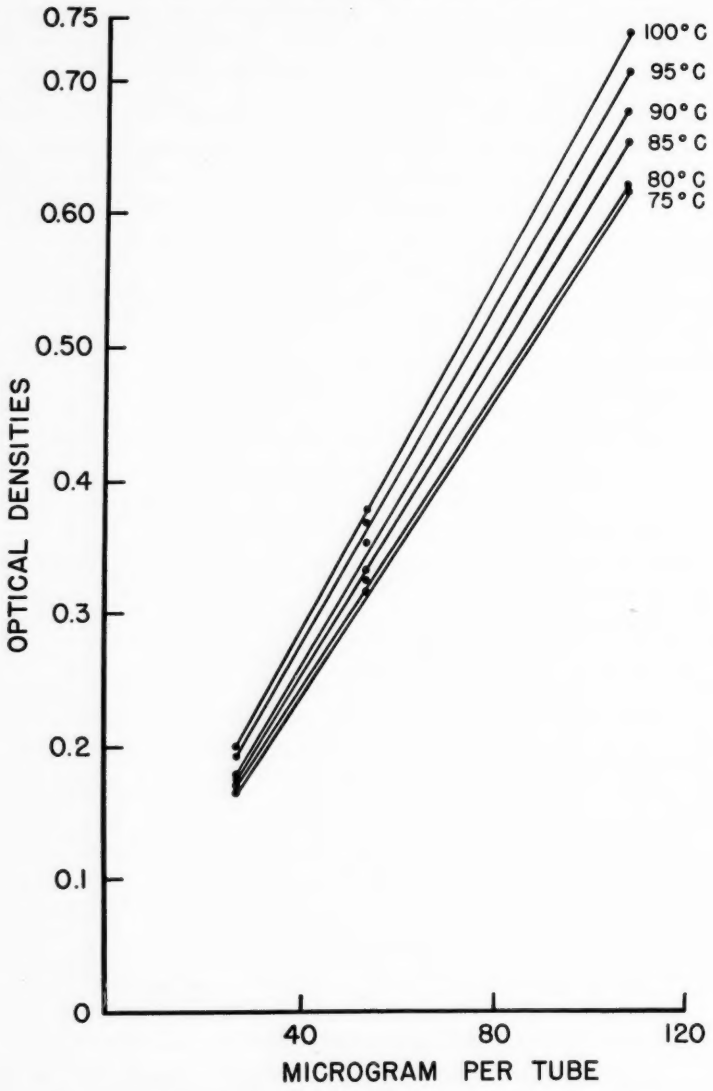

Fig. 1.—Effect of temperature upon sucrose color intensity.

stable in fifteen determinations. The optical densities of the invertase blanks in various situations (i.e., samples with no chromogen; with inulin; with sucrose; and with inulin and sucrose) were calculated as the difference between samples incubated with invertase and their exact counterparts without the enzyme. The optical densities of the invertase blanks were 0.015 ± 0.005 and 0.013 ± 0.005 for sixty plasma and forty-seven urine samples, respectively.

Table I shows the reproducibility of determinations and the coefficients of variation. The accuracy of this method was tested more critically by the recoveries of sucrose and/or inulin. These values are shown with their coefficients of variations in Table II. The recoveries of sucrose and inulin determined together were calculated as:

$$\frac{\text{O.D.I.S.}}{\text{O.D.I.} + \text{O.D.S.}} \times 100 = \text{recovery of sucrose plus inulin.}$$

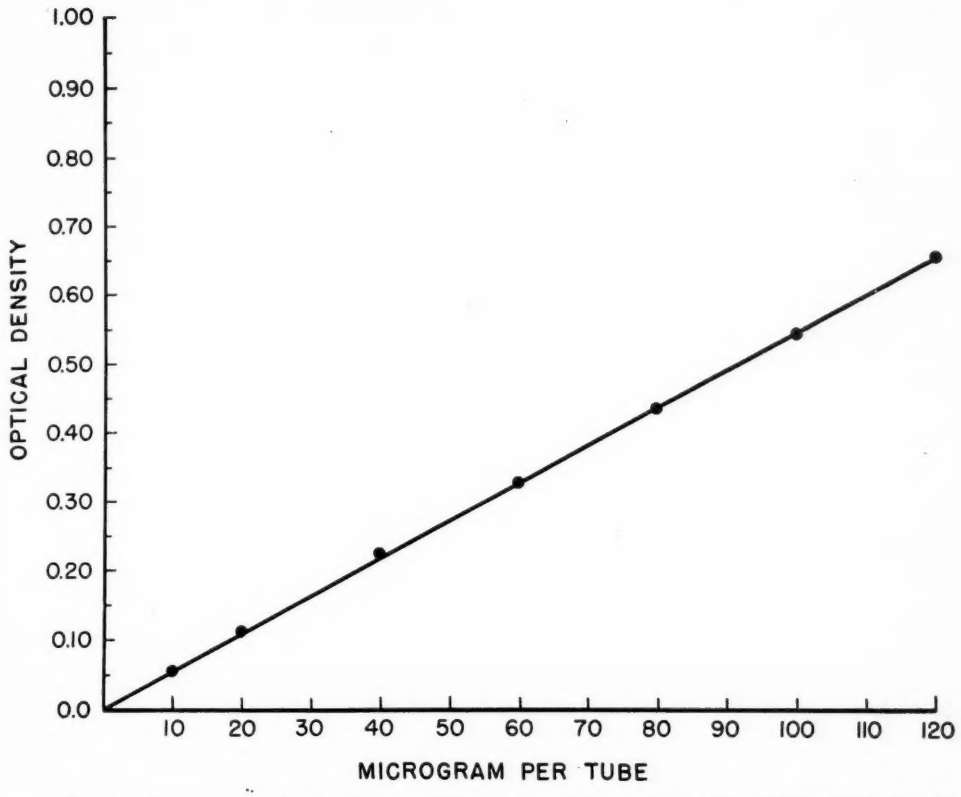

Fig. 2.—Sucrose standard curve. This range encompasses that encountered in the biologic material analyzed. Each point represents the mean of five determinations. The values with their coefficient of variation are: 10 ± 3.7 per cent; 20 ± 2.11 per cent; 40 ± 0.9 per cent; 60 ± 1.55 per cent; 80 ± 0.51 per cent; 100 ± 0.77 per cent; and 120 ± 1.15 per cent.

This recovery indicates no interference between the color reactions of sucrose and inulin. The recovery of sucrose was calculated as the difference in optical density of O.D.I.S. and O.D.I., thus:

$$\frac{\text{O.D.I.S.} - \text{O.D.I.}}{\text{O.D.S.}} \times 100 = \text{recovery of sucrose.}$$

The recoveries of sucrose and inulin following incubation with invertase are also shown in Table II. One hundred per cent recovery of inulin indicates the stability of inulin to invertase, and the complete inversion of sucrose is evidenced by essentially zero recovery. Coefficients of variations of the recovery values are given in Table II.

TABLE I. REPRODUCIBILITY OF OPTICAL DENSITY

DETERMINATION	SYMBOL	IN PLASMA		IN URINE	
		NO. DETERMINATIONS	COEFFICIENT OF VARIATION	NO. DETERMINATIONS	COEFFICIENT OF VARIATION
In	O.D.I	15	±1.76	9	±3.09
S	O.D.S.	35	±1.79	9	±3.87
In + S	O.D.I.S.	14	±2.00	9	±3.22
Incubation With Invertase					
In	$O.D.I._{Calc.}$	15	±3.11	9	±1.50
In + S	$O.D.I.S._{Inv.}$	15	±3.22	9	±2.13

In = Inulin.
S = Sucrose.
Calc. = Calculated value.

TABLE II. RECOVERIES OF SUCROSE AND INULIN

DETERMINATION	SYMBOL	IN PLASMA		IN URINE	
		PER CENT RECOVERY	COEFFICIENT OF VARIATION	PER CENT RECOVERY	COEFFICIENT OF VARIATION
In	O.D.I.	97.7	±1.80	99.7	±3.07
S	O.D.S.	101.6	±1.98	102.7	±4.29
In + S	$\frac{O.D.I.S.}{O.D.I. + O.D.S.}$	98.0	±1.84	101.6	±3.41
S	$\frac{O.D.I.S. - O.D.I.}{O.D.S.}$	98.0	±1.84	103.1	±3.41
Incubation With Invertase					
In	$\frac{O.D.I._{Inv.} - O.D._{Inv.}}{O.D.I.}$	100.3	±2.69	99.6	±2.40
In	$\frac{O.D.I.S._{Inv.} - O.D._{Inv.}}{O.D.I.}$	100.2	±2.73	100.3	±2.61
S	$\frac{O.D.S._{Inv.} - O.D._{Inv.}}{O.D.S.}$	0.4	±2.44	0.0	±3.04
S	$\frac{O.D.I.S._{Inv.} - O.D.I._{Inv.}}{O.D.S.}$	0.2	±1.74	0.3	±3.06

In = Inulin.
S = Sucrose.
Inv. = Invertase.

DISCUSSION

The determination of sucrose by the modified anthrone procedure offers the following distinct advantages: (a) The procedure measures sucrose directly. (b) Both the fructose and glucose fractions react to produce color. Resorcinol or reducing sugar tests determine only one fraction of the sucrose molecule. (c) The procedure is simple, highly reproducible, and accurate. (d) Anthrone has been demonstrated to give a stable color.

The advantages of the anthrone procedure for the determination of inulin have already been reported.[1] The use of an invertase preparation has proved to give reliable results, and offers the advantages of a low blank, with no correction necessary for yeast cell volume.

The data presented here for the simultaneous determination of inulin and sucrose have been pooled to give deviations caused by all conditions that may

be encountered in this procedure. Variations were evident from one day to the next. It must be borne in mind, therefore, that greater accuracy will be obtained by the use of standards and blanks from any particular day for the calculation of results on that day. This technique was followed routinely when this procedure was carried out in human studies.[5]

SUMMARY

An anthrone procedure has been developed for accurate determinations of sucrose and for the simultaneous determination of sucrose and inulin. Simultaneous determinations of these materials were accomplished by the measurement of optical densities of samples before and after inversion and subsequent digestion with NaOH.

A linear relationship existed between optical density and concentration of sucrose when the color was developed at temperatures from 75° to 100° C.

The commercial preparation of invertase employed in this study gave complete inversion of sucrose (i.e., no color caused by sucrose was evident after incubation). This preparation also gave low blank values.

The coefficients of variations for sucrose determinations were ± 1.79 per cent in plasma and ± 3.87 per cent in urine. Those for the determination of inulin were ± 1.76 per cent and ± 3.09 per cent in plasma and urine, respectively.

The recoveries of these materials after incubation with invertase gave 0.0 per cent recovery of sucrose and 100 per cent recovery of inulin.

The advantages of this technique are discussed.

We are grateful to Dr. Lawrence G. Raisz for his aid in the initiation of this project, and to Captain Jerry A. Stirman for his advice during its progress. The helpfulness of Colonel William H. Amspacher and the technical assistance of Ivan T. Stinson, Alfred J. Coscia, and Harley E. Sterling is appreciated.

REFERENCES

Young, M. K., and Raisz, L. G.: An Anthrone Procedure for Determination of Inulin in Biological Fluids, Proc. Soc. Exper. Biol. & Med. **80**: 771-774, 1952.

Raisz, L. G., Young, M. K., and Stinson, I. T.: Comparison of the Volumes of Distribution of Inulin, Sucrose and Thiosulfate in Normal and Nephrectomized Dogs, Am. J. Physiol. **174**: 72-78, 1953.

Keith, N. M., and Power, M. H.: The Urinary Excretion of Sucrose and Its Distribution in the Blood After Intravenous Injection Into Normal Men, Am. J. Physiol. **120**: 203-211, 1937.

Steinitz, K.: The Renal Excretion of Sucrose in Normal Man; Comparison With Inulin, Am. J. Physiol. **129**: 252-258, 1940.

Young, M. K., Stirman, J. A., and Prudden, J. F.: Human Fluid Compartments; The Simultaneous Volumes of Distribution of Sucrose, Inulin, Thiosulfate, Deuterium Oxide and T-1824. (To be published.)

THE DETERMINATION OF CYANIDE IN BIOLOGIC FLUIDS BY MICRODIFFUSION ANALYSIS

MILTON FELDSTEIN, M.A., AND NIELS C. KLENDSHOJ, M.D.
BUFFALO, N. Y.

MICRODIFFUSION analysis is admirably suited for the detection and estimation of small quantities of volatile substances. Conway[1] has pioneered the application of this method of analysis to the determination of such substances as ammonia, alcohol, carbon dioxide, volatile amines, and carbon monoxide. The Conway diffusion dish has become a standard laboratory item. This paper is concerned with the application of the microdiffusion technique to the estimation of cyanide in biologic fluids. The final colorimetric assay of cyanide is based on the procedure of Boxer and Rickards.[2]

MATERIALS AND METHODS

Reagents.—

a. One-tenth N NaOH.
b. One M NaH_2PO_4.
c. Twenty-five hundredths per cent solution of chloramine T (keep refrigerated).
d. Saturated aqueous solution of 1-phenyl-3-methyl-5-pyrazolone. Store in brown bottle. (Eastman Kodak Reagent, recrystallized from 95 per cent alcohol.)
e. One-tenth per cent solution in pyridine of bis-(1-phenyl-3-methyl-5-pyrazolone) prepared by boiling a mixture of 50 ml. ethylacetoacetate and 150 ml. phenylhydrazine under reflux until the solid bis-(1-phenyl-3-methyl-5-pyrazolone) separates. Filter and wash with hot alcohol. The pyridine solution is prepared fresh as needed.

The following reagents are prepared immediately prior to color development:

a. Chloramine T-phosphate reagent. To 1 part of 0.25 per cent chloramine T solution add 3 parts of 1 M NaH_2PO_4. Mix and keep in the ice bath.
b. Pyrazolone-pyridine reagent. To 5 parts of saturated aqueous solution of phenylmethylpyrazolone add 1 part of the bis-phenylmethylpyrazolone in pyridine.

Procedure.—It is essential that all glass apparatus be cleaned thoroughly in acid dichromate, rinsed in distilled water, and then oven dried. Tissue homogenates are prepared by blending a weighed portion of tissue in a Waring blendor with sufficient water to make a 1 to 5 dilution of tissue. Pipette an aliquot of blood plasma, urine, stomach contents, or tissue homogenate (usually 3 to 5 ml.) into the outer chamber of the standard Conway dish. To the central chamber add 2.0 ml. of 0.1 N NaOH to absorb the liberated hydrocyanic acid. The ground glass cover of the unit is smeared with petroleum jelly and placed on the dish; a small space is left at the edge for the final addition of 1 ml. 10 per cent sulfuric acid to the outer chamber. The lid is then moved into position to cover both chambers. The entire unit is rotated gently to mix the contents of the outer chamber, and then allowed to stand for two hours to permit complete absorption of hydrocyanic acid by the alkali in the central chamber.

From the University of Buffalo, School of Medicine, Division of Toxicology and the Department of Biochemistry, Buffalo General Hospital, Buffalo, N. Y.
Received for publication, March 21, 1954.

At the end of two hours, the lid is removed and 1.0 ml. of the alkali is transferred to a test tube. A blank consisting of 1.0 ml. of 0.1 N NaOH is prepared at the same time, and unknown and blank tubes are placed in an ice bath. To each tube add 0.2 ml. of chloramine T-phosphate reagent. The contents of the tubes are mixed, and allowed to stand for two to three minutes in the ice bath. This is followed by the addition of 3.0 ml. of pyrazolone-pyridine reagent. The contents of the tubes are mixed, and allowed to stand at room temperature for sixty minutes. The optical density of the blue color is then determined in a Beckman Model B spectrophotometer at 630 mμ with the blank set at zero density.

Standard solutions of cyanide containing 0.1 to 2.0 μg of cyanide per milliliter of 0.1 N sodium hydroxide are prepared and the color is developed as described above. A calibration curve relating optical density and concentration is obtained (Fig. 1), and the cyanide content of the material being analyzed is read from the curve. It can be seen from Fig. 1 that the Laws of Bouguer and Beer are obeyed in the range of concentration 0.1 to 2 μg of cyanide. This is equivalent to a concentration in 4 ml. biologic fluid of 5 to 100 μg per cent. The sensitivity can be increased by reducing the volume of sodium hydroxide in the center well of the Conway unit to 1.0 ml. The lower limit of sensitivity would then be 2.5 μg per 100 ml. sample or 0.1 μg in 4 ml. sample.

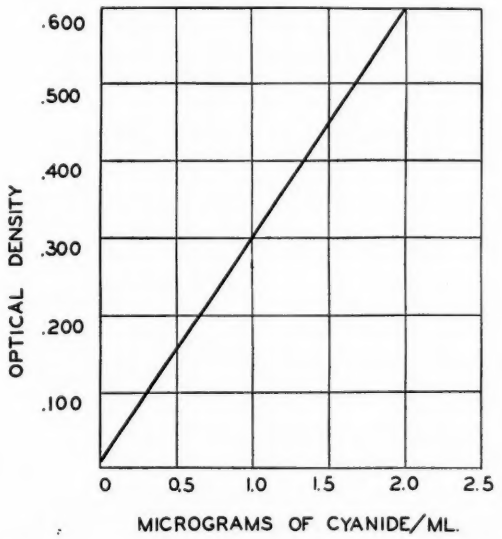

Fig. 1.—Concentration-Density curve for cyanide in 0.1 N NaOH. Wavelength = 630 millicrons; Beckman Model B Spectrophotometer; 1 cm. cells.

EXPERIMENTAL

In order to determine the optimum time required for the absorption of hydrocyanic acid, known quantities of cyanide were added to blood plasma, urine, and tissue homogenates, and these samples were then prepared for analysis, as described previously. The Conway units were allowed to stand for varying periods of time, after which the analysis for cyanide was completed, as described previously. Table I shows these results, and indicates that diffusion is complete in two hours. Recovery of added cyanide to samples of blood plasma, urine, and tissue homogenates is shown in Table II. The table indicates that recoveries range from 96 to 102 per cent.

TABLE I. THE EFFECT OF DIFFUSION TIME ON THE RECOVERY OF CYANIDE FROM PLASMA, URINE, AND TISSUE HOMOGENATES*

CYANIDE PRESENT (MICROGRAMS PER SAMPLE)	PER CENT RECOVERY TIME OF DIFFUSION (HOURS)					
	½	1	1.5	2	3	4
0.5	81	90	93	97	96	98
1.0	83	89	92	98	99	98
2.0	80	86	93	100	98	101

*These figures represent an average of several determinations, and are similar for plasma, urine, and tissue homogenates.

TABLE II. RECOVERY OF CYANIDE ADDED TO PLASMA, URINE, AND TISSUE HOMOGENATES

CYANIDE PRESENT (MICROGRAMS PER SAMPLE)	CYANIDE RECOVERED	
	MICROGRAMS	PER CENT
0.100	0.102	100
0.100	0.096	96
0.500	0.480	96
0.500	0.510	101
0.500	0.490	98
1.000	0.960	96
1.000	0.980	98
1.000	1.020	102
2.000	1.960	98
2.000	2.020	101

TABLE III. BLOOD PLASMA CYANIDE LEVEL OF NORMAL INDIVIDUALS AND OF PATIENTS WITH MULTIPLE SCLEROSIS

CASE NO.	CYANIDE LEVEL (MICROGRAMS PER CENT)
1*	1.5
2	9.7
3*	14.3
4	10.6
5	7.8
6*	9.2
7	1.2
8*	0.0
9	3.9
10	10.7
11*	8.6
12	3.1
13*	0.0
14	0.0
15	1.2
16	0.0

*Multiple sclerosis.

DISCUSSION

The possible presence of cyanide in normal blood and tissues has been the subject of many investigations. Gettler and Baine[3] reported no cyanide present when their method was used. Boxer and Rickards[4] report less than 0.4 μg per cent in biologic fluids, and describe the finding of traces of cyanide in respiratory air and urine.[5]

It has long been known that certain foods give rise to cyanide. These cyanogenetic foods contribute detectable quantities of cyanide to the blood and urine. The presence of a cyano group bound within the central coordina-

tion complex of the cobalt atom of vitamin B_{12} has been described by Brink and associates.[6] Fowler and colleagues have reported the finding of elevated cyanide levels in the blood of patients with multiple sclerosis.[7] Goldstein and Rieders have shown that an enzyme system capable of converting thiocyanate to cyanide is operative in dog and man.[8]

Normal blood plasma and urine and the plasma from cases of multiple sclerosis were assayed for their cyanide content as described previously. The results are listed in Table III. The results indicate that cyanide may normally be found in the blood and urine, and that there is no appreciable difference in the cyanide content of the plasma of normal individuals and the plasma of patients with multiple sclerosis.

Several cases of mild industrial exposure to cyanide fumes which did not terminate fatally were assayed by the present method with the results shown in Table IV. In each case where blood samples were obtained shortly after exposure, significantly higher than "normal" cyanide levels were obtained. These values return to "normal" in a short period of time.

TABLE IV. BLOOD PLASMA CYANIDE LEVEL OF INDIVIDUALS EXPOSED TO CYANIDE FUMES

CASE NO.	TIME AFTER EXPOSURE SPECIMEN WAS OBTAINED (HOURS)	½	1	2	4	8
		CYANIDE LEVEL (μg %)				
1		56	–	12	6	6
2		33	–	–	4	5
3		–	18	6	–	4
4		66	–	4	–	5
5		–	31	–	–	–

During the course of the investigation, it was found that recovery of cyanide added to freshly drawn samples of whole blood varied from 30 to 90 per cent. The lower recoveries were obtained when the added cyanide was in contact with red blood cells for one to five hours, and the higher recoveries were obtained when the samples were analyzed fifteen to twenty minutes after the addition of cyanide. Complete recovery of added cyanide was obtained when analysis of whole blood was started immediately after the addition of cyanide. Recovery of cyanide added to whole blood which had been standing for twenty-four hours was also complete. Evidence has been obtained indicating the presence of an active enzyme system in the red blood cells capable of removing added cyanide. This subject is being further investigated. The ability of tissue homogenates (liver, kidney, brain), plasma, and urine to remove cyanide is absent under the conditions employed here.

SUMMARY

A rapid, sensitive method for the determination of cyanide in biologic samples is presented. The method is based upon the diffusion of cyanide from the outer chamber of a Conway cell to the inner chamber, where it is absorbed in dilute alkali. The colorimetric estimation is performed by the method of Boxer and Rickards. Normal blood plasma cyanide levels are shown to vary from zero to 14 μg per cent. The role of cyanogenetic foods

and thiocyanate is mentioned as a possible source of normal cyanide. The plasma cyanide level of patients with multiple sclerosis does not differ substantially from the normal. Mild exposure to cyanide fumes results in increased blood levels if the sample is obtained shortly after exposure.

REFERENCES

1. Conway, E. J.: Microdiffusion Analysis and Volumetric Error, New York, 1950, D. Van Nostrand Co.
2. Boxer, G. E., and Rickards, J. C.: Chemical Determination of Vitamin B_{12}. II. The Quantitative Isolation and Colorimetric Determination of Millimicrogram Quantities of Cyanide, Arch. Biochem. **30**: 372, 1951.
3. Gettler, A. O., and Baine, J.: The Toxicology of Cyanide, Am. J. M. Sc. **195**: 182, 1938.
4. Boxer, G. E., and Rickards, J. C.: Determination of Thiocyanate in Body Fluids, Arch. Biochem. **39**: 292, 1952.
5. Boxer, G. E., and Rickards, J. C.: Chemical Determination of Vitamin B_{12}. IV. Assay of Vitamin B_{12} in Multivitamin Preparations and Biological Materials, Arch. Biochem. **30**: 392, 1951.
6. Brink, N. G., Kuehl, F. A., Jr., and Folkers, K.: Vitamin B_{12}: The Identification of Vitamin B_{12} as a Cyano-Cobalt Coordination Complex, Science **112**: 354, 1950.
7. Fowler, R. C., and Durbetaki, A. J.: Cyanide and Thiocyanate Determination in Multiple Sclerosis and Allied Demyelinating Disorders, Am. J. Physiol. **171**: 724, 1952.
8. Goldstein, F., and Rieders, F.: Formation of Cyanide in Dog and Man Following Administration of Thiocyanate, Am. J. Physiol. **167**: 47, 1951.